

Experimental and Clinical Studies on
the Function of
Antileukoprotease

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EXPERIMENTAL AND CLINICAL STUDIES
ON THE FUNCTION OF ANTILEUKOPROTEASE

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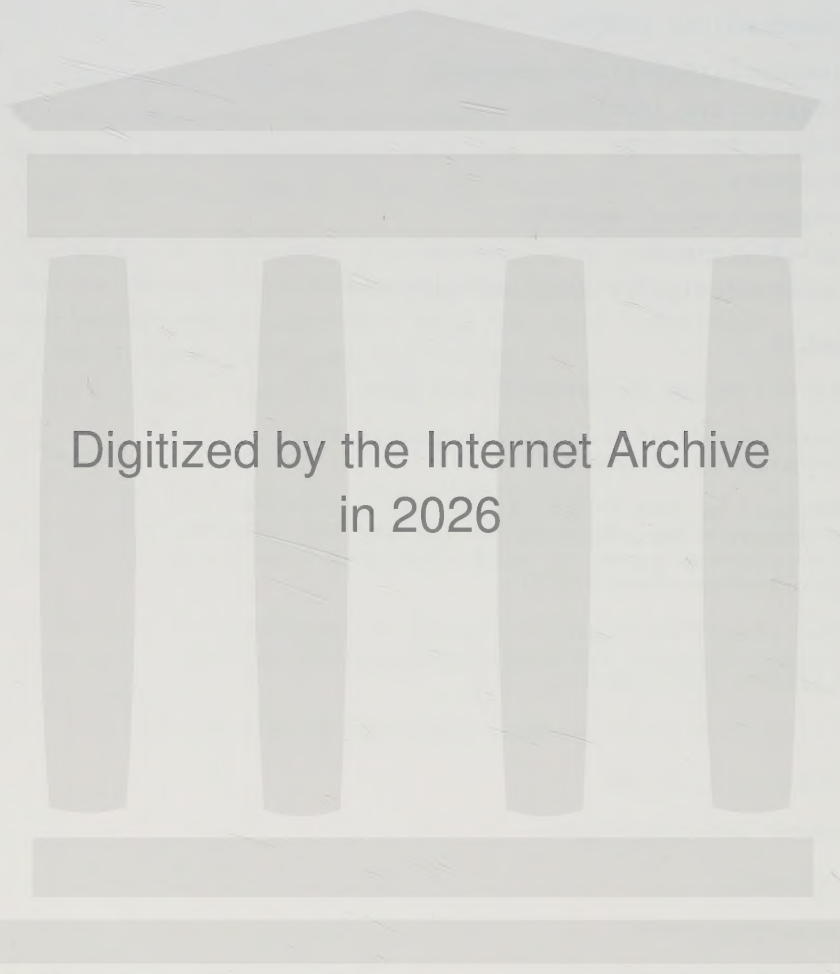
To Johanna and Emma

The present thesis is based on the following publications, which will be referred to by their Roman numerals.

- I. Fryksmark, U., Ohlsson, K., Polling, Å. & Tegner, H.
Distribution of antileukoprotease in upper respiratory mucosa.
Ann Otol Rhinol Laryngol 91, 268-271, 1982.
- II. Fryksmark, U., Ohlsson, K., Rosengren, M. & Tegner, H.
A radioimmunoassay for measurement and characterization of human antileukoprotease in serum.
Hoppe Seylers Z Physiol Chem 362, 1273-1277, 1981.
- III. Fryksmark, U., Ohlsson, K., Rosengren, M. & Tegner, H.
Studies on the interaction between leukocyte elastase, antileukoprotease and the plasma protease inhibitors α_1 -antitrypsin and α_2 -macroglobulin.
Hoppe Seylers Z Physiol Chem. In submission, 1983.
- IV. Ohlsson, K., Fryksmark, U. & Tegner, H.
The effect of cigarette smoke condensate on α_1 -antitrypsin, antileukoprotease and granulocyte elastase.
Eur J Clin Invest 10, 373-379, 1980.
- V. Fryksmark, U., Prellner, T., Tegner, H. & Ohlsson, K.
Studies on the role of antileukoprotease in respiratory tract diseases.
Eur J Respir Dis. In submission, 1983.

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INTRODUCTION

At the beginning of this century it had already been observed that purulent bronchial secretions displayed proteolytic activity. It was assumed that the proteolytic activity, designated "leucoprotease", originated from an enzyme produced in the polymorphonuclear leukocytes (101,102). At the same time, an antienzymatic activity, called "antileucoprotease", was found in human serum. This "antileucoprotease" was thought to pass from the blood into the inflammatory exudate, where it could counteract the "leucoprotease" (101,102).

The degree of proteolytic activity in bronchial secretions could be related to "purulency" and did not reflect any specific respiratory disease (70,71). Thus, proteolytic activity was not recovered in non-purulent sputum (71,72,102). Further studies of purulent sputum revealed three different types of proteolytic activity, indicating the presence of different proteases (72). The proteases were all active at neutral pH, but had different substrate specificities (68,69,72).

The characterization and origin of the proteolytic enzymes made it possible to divide them into two different groups. A. The metalloprotease, *specific collagenase*, located in the specific granulae of granulocytes (66,78). B. The serine proteases, *elastase* (7,44,87,114,124), *cathepsin G* (28,99,100, 105,114) and *neutral protease* (82,85), located in the azurophil granulae of granulocytes (95). In Table I some important characteristics are given.

The granulocyte proteases are released extra cellularly from the granulocyte granulae during phagocytosis (88). An inflammatory disease with a high concentration of granulocytes thus results in a local increase in proteolytic enzymes (81).

Several essential structures in the body, such as collagen and fibrin, are degraded by these proteases (Table I). In particular, the elastin degrading capacity of granulocyte elastase has been thoroughly investigated. Elastase digests arterial walls and basement membranes *in vitro* (47,48) and may produce haemorrhage *in vivo* (51,115). Human lung elastin is hydrolysed (50,68,115) and pulmonary emphysema can be induced by intra-

Table I. *Some characteristics of granulocyte proteases.*

Characteristics	Specific collagenase	Neutral protease	Elastase	Cathepsin G
	60,000 DA	76,000 DA	33,000 DA	25,000 DA
Molecular weight				
Metalloprotease	+	-	-	-
Serine protease	-	+	+	+
Localization				
azurophil granulae	-	+	+	+
specific granulae	+	-	-	-
Substrates				
collagen	+	+	+	+
fibrin	-	+	+	+
elastin	-	-	+	-
proteoglycans	-	+	+	+
complement				
component C3	-	+	+	+
component C5	-	-	+	+
clotting factors	-	-	+	+

References: 7,28,44,56,82,85,87,99,100,105,114,124,133

tracheal administration of elastase (51,115). Recently, an admixture of cathepsin G to elastase was reported to increase the elastolytic activity five-fold (12). This finding may play an important role in the development of pulmonary emphysema (12). The antibacterial activity of cathepsin G may also be of importance in the generation of inflammatory mediators (100,133).

The protease activity on the complement components C3 and C5 results in chemotactically active substances, which further increase the release of granulocyte proteases (56,133).

Proteolytic activity in bronchial secretions can also be derived from other sources than granulocytes. Thus, alveolar macrophages actively secrete proteolytic enzymes on phagocytotic stimulation (1). These enzymes have collagenolytic and elastolytic activities and have a neutral pH optimum. Two different elastases have been found in alveolar macrophages (140). One of

the elastases is a genuine macrophage elastase and belongs to the metalloproteases (138,140), whereas the other, belonging to the serine proteases, seems to be a phagocytised granulocyte elastase (38,140). The phagocytising of granulocyte elastase by alveolar macrophages may be an important route for the removal of this potentially pathogenic enzyme from the lung (140).

In addition, certain micro-organisms have been found to release proteases (141). Although we now know that most of these proteases differ from granulocyte proteases, there are a few fungi and bacteria, e.g. *pseudomonas aeruginosa* and staphylococci, which release proteases with elastase-like activity (55,138,141).

Free or unbalanced activity of the proteolytic enzymes would be injurious to any organism. The granulocyte proteases, released in connection with phagocytosis and cellular destruction, must therefore be neutralized. A defence mechanism, composed of inhibitors, inactivates the proteases by blocking their active sites (65). The plasma protease inhibitors, α_1 -antitrypsin, α_2 -macroglobulin and antichymotrypsin, constitute about 10 % of the total plasma protein content and are responsible for more than 90 % of the inhibitory capacity in plasma (65,76). They are the dominating inhibitors of granulocyte proteases (86,89,92,134). These inhibitors are also found in bronchial secretions (90,108,125). Since the protease inhibitory capacity of bronchial secretions was found to exceed that of the plasma protease inhibitors, it was suggested that bronchial secretions contained additional inhibitory factors (50,68). This hypothesis was corroborated by the finding that bronchial secretions contained an acid stable low molecular weight inhibitor, antileukoprotease. In addition, the inter- α -trypsin inhibitor, another plasma protease inhibitor, releases an inhibitory active fragment, which has been found in minute amounts in bronchial secretions (39,41).

GRANULOCYTE PROTEASE INHIBITORS

The inhibition specificity of the major granulocyte protease inhibitors in the respiratory tract is shown in Table II.

α_1 -Antitrypsin

α_1 -Antitrypsin is synthesized in the liver (65). It consists of more than 40 different variants with slightly varying amino acid sequences (20). This inhibitor is, on a molar basis, the quantitatively dominating protease inhibitor in plasma and the main plasma protease inhibitor in bronchial secretions (65,90, 119). α_1 -Antitrypsin inhibits serine proteases, i.e. the granulocyte neutral protease (89), cathepsin G (134) and elastase (86) in a molar ratio of 1:1. It is a more potent inhibitor of granulocyte elastase and chymotrypsin than of trypsin (5,103). It has therefore been proposed that the name α_1 -antitrypsin should be altered to α_1 -proteinase inhibitor (α_1 -PI) (103).

Both the esterolytic and the proteolytic activity of these enzymes are inhibited by α_1 -antitrypsin (65). The inhibitor is strongly associated to granulocyte elastase (36), but in dynamic systems, complexes of α_1 -antitrypsin and proteases dissociate (4,83). When once released from a protease, α_1 -antitrypsin does not retain all its inhibitory capacity (8,18,54) and a non-reactive α_1 -antitrypsin component has been observed in normal bronchial secretions (125).

α_1 -Antitrypsin responds as a general acute-phase reactant (65). Hereditary α_1 -antitrypsin deficiency is correlated with pulmonary emphysema (27,64), respiratory distress syndrom in premature babies (76) and liver diseases (123). An increased serum level of α_1 -antitrypsin has been reported in heavy smokers (67).

α_2 -Macroglobulin

α_2 -Macroglobulin has its main production site in the liver (65), although fibroblasts and macrophages have been shown *in vitro* to produce this inhibitor (77,139). It is a polyvalent inhibitor and enzymes belonging to all the four classes of endopeptidases are irreversibly bound by α_2 -macroglobulin (6). Recent investigations indicate a covalent binding of the proteases to α_2 -macro-

Table II. *Inhibition specificity of the major granulocyte protease inhibitors in the respiratory tract.*

Inhibitors	Molecular weight (DA)	Mean conc. in serum g/l	Proteases			
			Elastase	Cathepsin G	Neutral protease	Specific collagenase
α_1 -Antitrypsin	55.000	1.32	++	(+)	+	-
α_2 -Macroglobulin	725.000	2.0	+	++	++	++
Antichymotrypsin	69.000	0.5	-	+	-	-
Antileukoprotease	10.300	0.12×10^{-3}	++	+	-	-

+, ++ indicates inhibition of varying potency

- indicates no inhibiting effect

References: 52, 63, 65, 82, 85, 86, 89, 92, 98, 134, Paper II

globulin; this link may help to stabilize the complexes against dissociation (109). α_2 -Macroglobulin binds the granulocyte proteases, elastase and cathepsin G, in a molar ratio of 1:2 (86,134) and granulocyte neutral proteases in an equimolar ratio (89).

One major route for the elimination of proteases seems to be via the formation of α_2 -macroglobulin complexes, and the availability of α_2 -macroglobulin is of vital importance for protection against proteases, e.g. trypsin (4). A passive transfer from α_1 -antitrypsin to α_2 -macroglobulin may be an important step for at least some proteases (4,8). The cells of the reticulo-endothelial system and alveolar macrophages have been shown to take up and degrade protease- α_2 -macroglobulin complexes rapidly, *in vitro*, via a process involving transglutaminase (79,96). The elimination from the circulation is rapid, most of the complexes being phagocytised by the Kupffer cells of the liver ($T_{1/2} \sim 10$ min in man) (4,83).

Proteases bound to α_2 -macroglobulin lose their proteolytic activity but retain their esterolytic activity (6). The bound protease digests low molecular weight substrates and low molecular weight inhibitors may enter the complex (65). α_2 -Macroglobulin-bound granulocyte elastase has even been suggested to normally participate in the turnover of soluble precursors of elastin. In the presence of α_1 -antitrypsin deficiency it may contribute to the development of pulmonary emphysema (33).

The concentration of α_2 -macroglobulin in serum varies with age (65). The serum concentration is not increased by inflammation in man and α_2 -macroglobulin cannot therefore be included among the general acute-phase reactants. No deficiency state has been observed, but low values may be seen in critically ill patients (65).

Antichymotrypsin

Antichymotrypsin is a rather poor inhibitor of chymotrypsin (80), but is a potent inhibitor of cathepsin G (92), with which it forms complexes in a molar ratio of 1:1 (105,114,131). The inhibitor responds as an acute-phase reactant during bacterial infections and after a surgical trauma (2,65). Thus, it may

increase two-fold during the first eight hours after injury, which makes it comparable to C-reactive protein (2). Antichymotrypsin has been assumed to be involved in the defence mechanism of the mucosal membranes (108). It has been found both in normal and in purulent bronchial secretions (90,108,125). Bronchial secretions from children with cystic fibrosis and chronic bronchitis contain an increased level of antichymotrypsin (106). A high concentration of antichymotrypsin in colostrum suggests local production of this inhibitor (73).

The inter- α -trypsin inhibitor

The inter- α -trypsin inhibitor was given its name because it migrated between α_1 -antitrypsin and α_2 -macroglobulin on electrophoresis and was an efficient trypsin inhibitor *in vitro* (65). *In vivo* only about 3 % of the trypsin inhibitory capacity of plasma seems to be due to the inter- α -trypsin inhibitor (65). The inter- α -trypsin inhibitor is easily fragmented into small pieces by a variety of proteases and these fragments are usually active inhibitors (21). It has been suggested that the inter- α -trypsin inhibitor is a precursor to the secretory inhibitors, protecting the mucosal membranes against granulocyte proteases (65). One active fragment of the inter- α -trypsin inhibitor has been identified in bronchial mucus (39,41).

Antileukoprotease

The presence of antileukoprotease was first indicated by the finding that nasal and bronchial secretions contained an inhibitory capacity against trypsin and chymotrypsin, greater than that expected from the α_1 -antitrypsin content (40,42). Most of the inhibitor was recovered bound by proteases in the purulent bronchial secretions (43). The inhibitor could be released from the enzymes by perchloric acid treatment and was found to be acid stable (42). This inhibitor was responsible for about 70 % of the protease inhibitory capacity in bronchial secretions and was able to inactivate granulocyte proteases (42,43).

Antileukoprotease was given its name after its most conspicuous property, i.e. its high affinity for granulocyte elastase and cathepsin G (112). It is also active against trypsin, chymotrypsin

(42,91,127) and human pancreatic elastase (113), but it does not inactivate porcine pancreatic elastase (39,40,42). Antileuko-protease inhibits granulocyte elastase by forming stable complexes in a molar ratio of 1:1 (36,91). These antileukoprotease-elastase complexes may be dissociated by α_1 -antitrypsin or by acid treatment (36,40,98).

Isolation and characterization of antileukoprotease revealed that it had a molecular weight of 10.300 DA (98). In contrast to the plasma protease inhibitors it was not a glycoprotein (98).

Using an immunohistochemical method the presence of antileuko-protease has been demonstrated in different mucosal membranes, i.e. in the middle ear and respiratory tract mucosa, in the salivary glands and in the uterine cervical mucosa (13,17,93,126).

Simultaneously, an acid stable inhibitor, "Human-Seminalplasma-inhibitor" (HUSI-I), with a molecular weight of about 11.000 DA was isolated from human seminal plasma (111). This inhibitor formed strongly bound complexes with the granulocyte proteases, elastase and cathepsin G (111). From uterine cervical mucus another inhibitor, "Cervix-Uteri-Sekretinhibitor" (CUSI), revealed a striking similarity to HUSI-I, i.e. it was acid stable, and exhibited a strong inhibitory capacity against elastase and cathepsin G (113,136). The molecular weight was slightly above 11.000 DA (113,136). It also displayed immunological cross reactivity with HUSI-I, indicating a high degree of identity between the two inhibitors (29). Immunological cross reactivity is also found between HUSI-I and antileuko-protease (97) and between antileukoprotease and CUSI (112). The molecular weight and immunological cross reactivity indicate that they are identical proteins (113).

PURPOSES OF THE INVESTIGATION

The purposes of the present investigation were:

1. To localize antileukoprotease by using an indirect immuno-histochemical method.
2. To develop a radioimmunoassay for measurement of antileukoprotease in human serum, in bronchial secretions, and in other body fluids.
3. To study the stability of the complex between antileukoprotease and human granulocyte elastase and the interaction between the complex and the plasma protease inhibitors, α_1 -antitrypsin and α_2 -macroglobulin.
4. To investigate the inhibition of α_2 -macroglobulin-bound elastase by antileukoprotease.
5. To study the influence of cigarette smoke condensate *in vitro* on the function of antileukoprotease, α_1 -antitrypsin and granulocyte elastase.
6. To analyse antileukoprotease quantitatively and qualitatively in serum and sputum from patients with respiratory diseases in an attempt to elucidate the biological function of antileukoprotease.

METHODOLOGICAL ASPECTS

IMMUNOHISTOCHEMICAL PROCEDURE

The peroxidase-antiperoxidase (PAP) technique as described by Sternberger et al. (118) was used for the immunohistochemical identification of antileukoprotease (I). The procedure involves the use of a PAP complex, i.e. peroxidase coupled to a rabbit antibody against horseradish peroxidase. After the primary antibodies and the secondary unlabelled antirabbit IgG antibodies have been applied to the tissue section, PAP complex is added to react with the second antibody. For the light microscopical identification of peroxidase, diaminobenzidine is used as a substrate.

Tissue specimens were fixed in buffered formalin solution and embedded in histowax. Sections were cut at 5 μ , dewaxed and hydrated in graded ethanol solutions. Following rinsing in 0.05 M Tris-HCl buffer, pH 7.6, containing 0.15 M NaCl, the sections were incubated in a solution of pepsin and treated with normal non-immune swine serum to reduce background staining (75,104). Specific rabbit antiserum against antileukoprotease was applied for 30 minutes. The antigen-antibody reaction was revealed by swine-antirabbit IgG, followed by 30 minutes of incubation with PAP complex. Peroxidase was made visible by exposure to 3,3'-diaminobenzidine and hydrogenperoxide. After counterstaining with the PAS-method or hematoxylin, sections were dehydrated and mounted in Pertex. All steps were carried out at room temperature in a humidified chamber.

Compared with a previous investigation (126), the procedure was altered in the following way:

1. Buffered formalin solution was used instead of Bouin's solution, which contains picric acid.
2. Sections were incubated in a solution of pepsin and then treated with normal swine serum to reduce background staining (75,104).
3. The washing procedure was extended (104).
4. Higher dilutions of antisera were used (1:1000-2000).

5. Sections were counterstained with the PAS-method or with hematoxylin.

Unspecific staining is common in immunohistochemistry. For testing the specificity of the staining the antiserum was inactivated before its application to the sections. Antisera can be inactivated either by addition of antigen in excess or by elimination of the specific antibodies by e.g. affinity chromatography (132). In the present study (I), sections incubated with antiserum, previously adsorbed with human antileukoprotease, served as controls.

RADIOACTIVE LABELLING

Using a lactoperoxidase method (129), antileukoprotease and elastase were labelled with ^{125}I (II,III). Unbound iodide was removed from the reaction mixture by gel-filtration on a Sephadex G-50 column. The fraction containing the highest counts was divided into small aliquot portions and frozen at -70°C .

Radioactive labelling causes a number of secondary changes in the protein that may affect its behaviour in an assay system. With the present labelling method labelled proteins retain their immunological activity and more than 80 % of the antibody-binding capacity (129).

RADIOIMMUNOASSAY

In a radioimmunoassay a radioactive labelled antigen (tracer) competes with the antigen in the sample for the binding sites of a constant amount of antibodies. The tracer and the antibodies are added to the sample and the mixture is incubated. The concentrations of antigens and antibodies are usually too low to precipitate. Therefore, a precipitating agent, i.e. a second antibody directed against the first antibody is added to the sample. After centrifugation, the soluble part of the sample is decanted and the radioactivity of the precipitate is measured. The larger the amount of antigen present in the sample, the smaller the amount of tracer which will be recovered in the final antigen-antibody precipitate. In order to measure the amount of antigen in the sample, the radioactivity obtained from

the precipitates of the samples is compared with the radioactivity of precipitates from standard solutions with known antigen concentrations.

In the present study, antileukoprotease was labelled with ^{125}I to a specific activity of about 2600 MBq/mg (II). The properties of the labelled tracer were tested in precipitation experiments. With an antiserum against antileukoprotease in a dilution of 1:8000, 82 % of the tracer was precipitated. An antiserum dilution of 1:30.000, yielding about 78 % precipitation of the radioactivity, was chosen to obtain an optimal standard curve in the range of desired measurement. The tracer was used at a concentration of 1.75 ng/ml, corresponding to about 50.000 cpm/200 μl .

The specificity of a radioimmunoassay is the ability to measure a specific compound. One way to study the specificity is to examine the effect of dilution of standard and sample with a buffer. An equal dilution of standard and sample should result in the same recovery, which can be illustrated by plotting dilution curves. Similar immunoreactivity is indicated by parallel curves, whereas differences in the immunoreactivity between the antigen used as standard and the antigen in the samples are expressed by non-parallel curves.

The sensitivity of a radioimmunoassay may be defined as the least amount of antigen distinguishable from zero (135). In the present study substances down to concentrations below 2 $\mu\text{g/l}$ could be measured. This sensitivity made it convenient for the determination of minute amounts of antileukoprotease in human serum (II,V) and in bronchial secretions (V).

A previously characterized radioimmunoassay (84) was used to examine granulocyte elastase in bronchial secretions (V).

ANTISERA

The validity of the immunohistochemical and radioimmunoassay results depends on the quality of the antisera used.

Antiserum against antileukoprotease and the IgG fraction of this antiserum were produced as previously described (91). These antisera were tested against antileukoprotease-containing

materials by immunoelectrophoresis, resulting in a single line of precipitation (91).

Antisera against α_1 -antitrypsin, α_2 -macroglobulin, antichymotrypsin and orosomucoid are available at our laboratory.

IMMUNOCHEMICAL METHODS

Radial immunodiffusion ad modum Mancini (74) was used to identify antileukoprotease in complex with ^{125}I -elastase (III). Electro-immunoassay according to Laurell (62) was performed to identify cigarette smoke condensate treated α_1 -antitrypsin (IV) and to localize α_1 -antitrypsin and α_2 -macroglobulin after gel-filtration of human serum (III,V). This method was also used for quantitative analysis of α_1 -antitrypsin, antichymotrypsin and orosomucoid (V).

The sensitivities for these two methods are similar but the specificity for the electroimmunoassay is considered to be higher (62). The precipitates of the electroimmunoassay reflect more properties of the antigen-carrying molecules than the precipitates of radial immunodiffusion. The radial immunodiffusion is, however, more appropriate for molecules below 40.000 DA and this method was therefore used for the identification of antileukoprotease (62).

Agarose gel electrophoresis (53), immunoelectrophoresis (110) and crossed immunoelectrophoresis (35) were used to study the affect of cigarette smoke condensate on human serum, α_1 -antitrypsin and antileukoprotease (IV). These methods are convenient for investigations of alterations and heterogeneities in the protein molecules.

GEL-FILTRATION

Sephadex is a chromatographic material, capable of separating substances according to molecular size. Sephadex G-75 and G-200 were used to obtain optimal separation within the desired range of molecular weight. Gel-filtration on Sephadex columns was performed in order to separate granulocyte elastase and its inhibitors, i.e. α_1 -antitrypsin, α_2 -macroglobulin and antileukoprotease, and also to study the complex formation of granulocyte

elastase and antileukoprotease in human serum and in bronchial secretions (III,V).

DETERMINATION OF ELASTASE ACTIVITY

The granulocyte elastase activity and the inhibition capacity of elastase by antileukoprotease (III,IV,V) was measured spectrophotometrically at 37° C, using N-succinyl-L-alanyl-L-alanyl-L-alanine-p-nitroanilide (Suc(Ala)₃NA) as a substrate (9). This method has a high specificity for elastase and enzyme concentrations as low as 0.05 µg/ml may be accurately assessed (9,94).

RESULTS

LOCALIZATION OF ANTILEUKOPROTEASE (I)

Human tracheal mucous membranes were obtained from patients undergoing tracheotomy. Maxillary sinus mucosa was acquired from healthy subjects operated on because of facial deformities.

Positive staining for antileukoprotease was limited to the serous parts of the seromucous glands of tracheal and maxillary sinus mucosa. The presence of antileukoprotease could not be demonstrated in the PAS positive cells or in any cell of the ciliated epithelium. Control sections were incubated with specific antiserum, previously adsorbed with antileukoprotease. In the control sections the positive staining was absent confirming the specificity of the reaction for antileukoprotease.

DEVELOPMENT OF A RADIOIMMUNOASSAY FOR ANTILEUKOPROTEASE (II)

The specificity of the radioimmunoassay for antileukoprotease was tested with dilution curves for antileukoprotease and for six different serum samples. The curves were all parallel. The sensitivity of the assay was $2.0 \mu\text{g/l}$. The concentration of antileukoprotease in sampled sera from healthy blood donors was $126 \pm 19.5 \mu\text{g/l}$. The results obtained had a coefficient of variation within the assay of 5.96 %. The recovery of exogenous antileukoprotease added to normal serum was about 98 %. Gel-filtration of normal serum resulted in one single peak of antileukoprotease, corresponding to the molecular weight of free antileukoprotease. In serum, where the elastase inhibitors were saturated by the addition of granulocyte elastase, antileukoprotease was recovered in the fractions corresponding to antileukoprotease-elastase complexes, indicating the presence of a free and active inhibitor. Gel-filtration of this material revealed a change in the immunoreactivity of antileukoprotease after binding to elastase. This finding was further supported when antileukoprotease-elastase complexes were tested in dilution series. The resultant curves showed a pattern indicating altered and diminished immunoreactivity of the complexed antileukoprotease.

STUDIES ON THE STABILITY OF THE ANTILEUKOPROTEASE-ELASTASE
COMPLEX AND THE INHIBITION OF ELASTASE BOUND TO α_2 -MACROGLOBULIN
BY ANTILEUKOPROTEASE (III)

Experimental procedures

1. Using a lactoperoxidase method (129) elastase was labelled with ^{125}I . An equimolar complex of antileukoprotease and ^{125}I -elastase was produced. The complex solution was subjected to gel-filtration after 10 minutes and 24 hours of incubation. The fractions containing the highest radioactivity, indicating ^{125}I -elastase, coincided with the fractions containing antileukoprotease, located with radial immunodiffusion. These fractions were pooled and rechromatographed after another 24 hours of incubation.
2. To study the stability of equimolar complexes of antileukoprotease and elastase, two complexes were produced, one consisting of ^{125}I -antileukoprotease-elastase and the other of ^{125}I -elastase-antileukoprotease. The complexes were mixed with different serum samples. The mixtures were subjected to gel-filtration immediately and after 24 hours of incubation and the radioactivity of the fractions was measured. α_1 -Antitrypsin and α_2 -macroglobulin were localized by electroimmunoassay and antileukoprotease with radial immunodiffusion. In addition, the inhibition of elastase activity was tested by the low molecular weight substrate $\text{Suc(Ala)}_3\text{NA}$ (9).
3. The possible inhibition of α_2 -macroglobulin-bound elastase by antileukoprotease was studied. Elastase- α_2 -macroglobulin complexes were produced in a molar ratio of 1:1 and 2:1. After addition of increasing amounts of antileukoprotease to the complexes, the mixtures were incubated for 1 hour, 4 hours or 24 hours. Elastase activity of the fractions was studied with $\text{Suc(Ala)}_3\text{NA}$ as a substrate.

Results

1. Rechromatography of the equimolar ^{125}I -elastase-antileukoprotease complexes after 24 hours of incubation showed that the radioactivity eluted in one single peak, containing ^{125}I -elastase-antileukoprotease. Antileukoprotease, located with radial immunodiffusion was recovered only within the ^{125}I -elastase-containing fractions. No free elastase activity was detectable using $\text{Suc}(\text{Ala})_3\text{NA}$ as a substrate. Thus, there were no signs of dissociation of the ^{125}I -elastase-antileukoprotease complex.
2. Analysis of the fractions, recovered from gel-filtration of the antileukoprotease-elastase complexes in mixture with human serum, revealed a rapid dissociation of the antileukoprotease-elastase complexes. When the mixture was immediately subjected to gel-filtration, more than 80 % of the antileukoprotease was found in the fractions corresponding to the free inhibitor and about 4 % in the α_2 -macroglobulin-containing fractions. About 90 % of the ^{125}I -elastase was recovered bound to α_1 -antitrypsin and about 10 % was bound to α_2 -macroglobulin. With an incubation time of 24 hours the corresponding figures for elastase bound to α_1 -antitrypsin and to α_2 -macroglobulin were 75 % and 25 %, respectively. Antileukoprotease was now recovered only in the fractions corresponding to free antileukoprotease and to α_2 -macroglobulin, indicating complete dissociation of the antileukoprotease-elastase complexes. In addition, some elastase activity was detected in the α_2 -macroglobulin-containing fractions, suggesting α_2 -macroglobulin-bound elastase, still active on $\text{Suc}(\text{Ala})_3\text{NA}$.
3. The rate and degree of inhibition of the α_2 -macroglobulin-elastase by antileukoprotease were found to be lower when α_2 -macroglobulin was saturated (1:2) with elastase than when the complexes were incompletely saturated (1:1). An increased incubation time diminished the quantities of antileukoprotease needed to reach equilibrium. However, even if a great excess of inhibitor was used in combination with long incubation time, complete inhibition did not occur. The greatest inhibition, reached when α_2 -macroglobulin was incompletely saturated with elastase, was about 75 %.

THE EFFECT OF CIGARETTE SMOKE CONDENSATE ON α_1 -ANTITRYPSIN, ANTILEUKOPROTEASE AND GRANULOCYTE ELASTASE (IV)

General aspects

Cigarette smokers with chronic bronchitis often develop pulmonary emphysema (3,117). Cigarette smoke has been reported to suppress the elastase inhibitory capacity of α_1 -antitrypsin in serum and in bronchial secretions (15,30,45,49).

To further elucidate these observations the effect of cigarette smoke condensate on α_1 -antitrypsin, antileukoprotease and granulocyte elastase, was studied. Cigarette smoke condensate was obtained from the research laboratory of the Swedish Tobacco Company. The cigarette smoke condensate was dissolved to a weight volume (w/v) of 20 %, in dimethylsulphoxide. Different amounts of the solution were incubated with normal human serum, α_1 -antitrypsin, antileukoprotease and granulocyte elastase, respectively.

Results

After addition of cigarette smoke condensate to human serum a distortion of the electrophoretic pattern was observed on agarose gel electrophoresis. With increasing amounts of smoke condensate the α_1 -antitrypsin band disappeared, other protein bands became progressively more diffuse and finally, with the highest amount of smoke condensate added, only the albumin band was present. The addition of smoke condensate solution to pure α_1 -antitrypsin resulted in an increased mobility and a pronounced band widening. Crossed immunoelectrophoresis of the reaction mixture exposed a new α_1 -antitrypsin component. This component increased with the amount of smoke condensate added, while the original α_1 -antitrypsin precipitate decreased.

Also antileukoprotease showed progressive changes after incubation with smoke condensate. The most obvious change was a decreased cationic mobility.

In addition, the inhibitory capacity of α_1 -antitrypsin and antileukoprotease towards granulocyte elastase was diminished in a time- and dose-dependent manner by smoke condensate.

Cigarette smoke condensate was also found to affect elastase in a dose-dependent way. The elastolytic as well as the esterolytic activities of elastase were suppressed by prolonged incubation with smoke condensate and the elastin degrading activity was decreased to zero by increasing amounts of smoke condensate.

CLINICAL ASPECTS ON ANTILEUKOPROTEASE (V)

Serum samples

Serum samples from patients with pneumonia were drawn every two days during their hospital stay. The patients all had a history consistent with lower respiratory tract infection and their chest radiographs showed fresh pulmonary shadowing. "Convalescence" sera were obtained, at the earliest, one month after discharge from hospital.

Sera from a control group, composed of patients with cholecystolithiasis, were drawn the day before and during three consecutive days after cholecystectomy.

Bronchial secretions

Bronchial secretions were collected from patients with chronic bronchitis staying in hospital, suffering an exacerbation of their disease, and from a group of out-patients with chronic bronchitis. Ultracentrifugation at $240.000 \times g$ at $4^{\circ} C$ for three hours was carried out in order to separate the sol phase from the gel phase of the secretions (107).

Results

Patients with pneumonia had an increased circulating level of antileukoprotease on admission to hospital ($p < 0.002$). The values decreased between day one and day five. Their convalescence sera displayed an additional decrease ($p < 0.002$), down to the pre-operative values of the control patients. Like antileukoprotease, the general acute-phase reactants increased in serum in patients with pneumonia, but the levels did not decrease during the first five days. The circulating levels of the general

acute-phase reactants were normal in the convalescence sera. The patients with cholecystolithiasis showed increased serum concentrations of the general acute-phase reactants after cholecystectomy ($p < 0.01$). In contrast, the circulating level of antileukoprotease did not increase in these patients after surgery.

Gel-filtration of sera from patients with pneumonia revealed antileukoprotease in a free and active form. Elastase was recovered in the fractions corresponding to α_1 -antitrypsin, indicating that elastase was α_1 -antitrypsin-bound in these sera.

The complex formation of elastase and antileukoprotease was studied in the sol phase of purulent bronchial secretions. Free elastase activity was found in three out of eight analysed secretions. All antileukoprotease was recovered bound to elastase in these samples. In contrast, the sputa without elastase activity displayed large amounts of free antileukoprotease in addition to elastase-antileukoprotease complexes.

GENERAL DISCUSSION

Antileukoprotease is present in high concentrations in bronchial secretions (42,91,125), indicating a local production of the inhibitor. This indication was upheld by the results from a previous immunohistochemical study, demonstrating the presence of antileukoprotease in the seromucous glands and in the ciliated epithelium of the tracheal mucosa (126). In the present study, using an improved immunohistochemical method, antileukoprotease was shown to be confined to the serous parts of the seromucous glands of tracheal and maxillary sinus mucosa. Neither the PAS positive cells of the seromucous glands nor the ciliated cells of the epithelium of the mucosa from these regions displayed immunoreactive antileukoprotease (I).

The findings are in agreement with those of Kramps et al. (59), who reported on the occurrence of antileukoprotease in the serous parts of the seromucous glands in the respiratory tract mucosa. Recently, antileukoprotease has also been shown to be present in the serous parts of the salivary glands (22,93). In addition, immunohistochemical studies have demonstrated antileukoprotease in PAS positive goblet cells in the middle ear mucosa (13), in the secretory cells in the epithelium of the bronchioles (22,93) and in the uterine cervical mucosa (17).

The vast area of the respiratory tract mucosa, compared with other antileukoprotease-containing mucous membranes, probably makes it the main productive site of antileukoprotease. However, the concentration of antileukoprotease varies depending on from which part of the respiratory tract the secretions are obtained (31). Thus, according to a recent study, bronchial lavage fluid from exclusively the lower part of the respiratory tract contains no demonstrable antileukoprotease-like activity, whereas its presence can be readily demonstrated in secretions from the bronchial tree (31,125). In the alveolar area α_1 -antitrypsin is the dominating inhibitor (31). The immunohistochemical demonstration of antileukoprotease in the secretory cells of the epithelium of the bronchioles indicates a function for antileukoprotease also in the lower respiratory tract (93). Consistently, the concentration of antileukoprotease in bronchial secretions is in great excess of the level in serum (91,125) (II).

It was therefore anticipated that antileukoprotease, present in human serum in trace amounts, is the result of a spill-over from the respiratory tract. This is the case with other secretory proteins, e.g. trypsinogen and the pancreatic secretory trypsin inhibitor (PSTI) (11,24). Thus, a radioimmunoassay, sensitive enough to show the presence of antileukoprotease in human serum and other fluids, was developed (II).

Many steps in an immunoassay is imprecise leading to an overall uncertainty in the derived assay value (135). The assay results of the radioimmunoassay for antileukoprotease were reproducible and the measured values had an acceptable coefficient of variation within the assay (II). The sensitivity of the radioimmunoassay was $2 \mu\text{g/l}$, which was adequate to measure antileukoprotease in sera from healthy individuals. Gel-filtration analysis of human serum showed antileukoprotease in a free and active form with retained capacity to bind elastase (II). The formation of complexes of antileukoprotease and elastase seemed to alter and diminish the immunoreactivity, as shown by analysis of dilution curves for the free and complexed inhibitor. Quantification of antileukoprotease, when in complex with elastase, revealed values of about 30 % of the true antileukoprotease content. The binding of elastase to antileukoprotease thus probably resulted in a blockage of antigenic sites on the inhibitor.

The presence of antileukoprotease in a free form in human serum was first somewhat confusing, since the antileukoprotease-elastase complex has been shown to be very stable (36) (III). The equilibrium dissociation constant (K_1) of the antileukoprotease-elastase complex is among the lowest for natural protease inhibitors (36) and the antileukoprotease-elastase complex does not dissociate during 24 hours of incubation (III). Complexes between other acid stable low molecular weight inhibitors and proteases have, however, been shown to dissociate spontaneously (61). Thus, the equimolar PSTI-trypsin complexes are partially dissociated after 16 hours of incubation (25).

The addition of the antileukoprotease-elastase complex or the PSTI-trypsin complex to human serum, results nevertheless in

similar dissociation patterns (25) (III). Both complexes dissociate rapidly and in both cases the major part of the inhibitors is recovered in a free form. A few percent of antileukoprotease, or PSTI, are bound in the α_2 -macroglobulin fraction. Elastase from the antileukoprotease-elastase complex is mainly bound to α_1 -antitrypsin and to a minor degree bound to α_2 -macroglobulin, while two thirds of the trypsin form complexes with α_2 -macroglobulin (25) (III).

The rapid dissociation of the antileukoprotease-elastase complex in human serum can probably be explained kinetically as a result of the more rapid binding of elastase by α_1 -antitrypsin ($K_{\text{ass}} 6.5 \cdot 10^7 \text{M}^{-1} \cdot \text{s}^{-1}$) as compared with antileukoprotease ($K_{\text{ass}} 1.1 \cdot 10^7 \text{M}^{-1} \cdot \text{s}^{-1}$) (36). Through this interaction, α_1 -antitrypsin forms complexes with elastase and consequently antileukoprotease is recovered in a free form in serum. Furthermore, no free elastase was found, but some elastase activity on Suc(Ala)₃NA as a substrate was displayed in the α_2 -macroglobulin-containing fractions (III). This finding is consistent with the fact that proteases bound to α_2 -macroglobulin retain their proteolytic activity against small molecules (6,65). Not only elastase but also antileukoprotease was revealed in the α_2 -macroglobulin-containing fractions. This raised the question of an inhibition of elastase bound to α_2 -macroglobulin by antileukoprotease. α_2 -Macroglobulin-elastase complexes in a molar ratio of 1:1 and 1:2 were both partially inhibited by antileukoprotease in a slow time-dependent way. The inhibition was more complete when the α_2 -macroglobulin-elastase complex was unsaturated (1:1), than when it was saturated (1:2) (III). The inhibition rate was, however, of the order of hours which might argue against any biological role of the antileukoprotease interaction with the elastase- α_2 -macroglobulin complexes. These results are in accordance with a study on α_2 -macroglobulin-bound trypsin inhibited by the soybean trypsin inhibitor (STI) (10). The activity of α_2 -macroglobulin-bound trypsin in a molar ratio of 1:1 was reported to be more susceptible to inhibition by STI than the complex in a molar ratio of 1:2 (10). A sterical hinder to the reaction of STI with the second trypsin molecule was suggested (10). This may explain why inhibition by antileukoprotease is more complete when α_2 -macroglobulin is incompletely

saturated by elastase. These observations are in agreement with the behaviour of other low molecular weight inhibitors like PSTI and aprotinin and makes antileukoprotease comparable to those inhibitors (23,25,34).

Antileukoprotease and α_1 -antitrypsin are the main inhibitors of granulocyte elastase in the respiratory tract as judged from the inhibitory spectrum of bronchial secretions (31,42,91,125). A diminished inhibitory capacity or an increase of granulocyte elastase may lead to free elastase activity. The clinical consequences of decreased inhibitory capacity were first elucidated by Laurell and Eriksson, who found that patients with α_1 -antitrypsin deficiency may develop pulmonary emphysema (27,64). Several experimental studies were later performed to evaluate the role of protease activity in pulmonary connective tissue destruction (37,51,115,116). These findings support the theory that granulocyte elastase, possibly in connection with macrophage elastase (139), are the major causes of the pulmonary emphysema. The significance of a local defence is made evident by the observation that experimentally induced emphysema in dogs is initiated via the air-lung interface and not via the intra-vascular route (137). In addition, the presence of granulocyte elastase has been immunologically demonstrated in connective tissue interstitium of freshly excised canine lung (51).

α_1 -Antitrypsin deficiency and cigarette smoking must be considered risk factors which may contribute to the emphysematous disease (3). The production and/or release of granulocyte and macrophage elastase is stimulated by cigarette smoke (58). Furthermore, cigarette smoke condensate suppresses the elastase inhibitory capacity of human serum (15,45). This is supposed to be due to an alteration of α_1 -antitrypsin (15,45). These observations are confirmed and extended in the present study (IV). An effect on most serum proteins was obtained when human serum was incubated with cigarette smoke condensate. α_1 -Antitrypsin displayed a pronounced time- and dose-dependent change and a decreased inhibitory capacity against elastase. Crossed immunoelectrophoresis revealed α_1 -antitrypsin components with varying immunoreactivity (IV). Current investigations of the effect of cigarette smoke on α_1 -antitrypsin support these findings (16,30,49,122). Bronchial lavage fluids from smokers exhibit a

changed α_1 -antitrypsin and a reduced elastase inhibitory capacity (16,30,49,122).

In the present study (IV) it was shown that antileukoprotease reacted in much the same way as α_1 -antitrypsin when exposed to cigarette smoke condensate; viz. altered electrophoretic properties and a time- and dose-dependent decrease of the elastase inhibitory capacity. As antileukoprotease is the dominating elastase inhibitor of the ciliated epithelium in the respiratory tract these cigarette smoke induced effects may have a deleterious effect on the integrity and function of the mucosa in smokers.

Another interesting finding was the decreased activity of granulocyte elastase when it had been incubated with cigarette smoke condensate (IV). This observation has since been confirmed by two other independent groups (26,46). Although they used an aqueous solution of cigarette smoke instead of cigarette smoke condensate, the decreased activity of elastase was so obvious that it cannot be neglected. One of the reports stated that the serum elastase inhibitory capacity is more susceptible to inactivation by aqueous solution of cigarette smoke than is granulocyte elastase (46). Thus, the influence of cigarette smoke on enzymes and inhibitors in the respiratory tract is probably more complex than initially anticipated. Studies dealing with analyses of the protease-antiprotease balance in bronchial lavage fluids of smokers and non-smokers would probably help to further elucidate these problems.

Recently, the change in α_1 -antitrypsin induced by cigarette smoke was suggested to be due to an oxidation of a methionine residue (16,19,130). Furthermore, the biological oxidant from neutrophil granulae, myeloperoxidase, may contribute to the oxidation of α_1 -antitrypsin (130). Different ways to counteract the oxidation have been discussed (32), e.g. by giving antioxidantia like vitamin C and E (32). An alternative would be to stop smoking.

The plasma protease inhibitors, α_1 -antitrypsin and antichymotrypsin, respond to an infection in the respiratory tract with raised serum concentrations (65). These concentrations are also increased in infected sputa and for antichymotrypsin to a higher level than can be explained by diffusion from sera alone (120).

In bronchial secretions, antileukoprotease is the dominating inhibitor (42,125). During the course of an infectious respiratory disease there is possibly an increased production of antileukoprotease which might be reflected in the serum concentration. This was shown in the present study (V), in which the circulating level of antileukoprotease increased significantly in patients with pneumonia. During recovery the serum level of antileukoprotease decreased faster than that of the general acute-phase reactants (α_1 -antitrypsin and antichymotrypsin). Surgical trauma did not affect the serum level of antileukoprotease as distinguished from the general acute-phase reactants. These results indicate that antileukoprotease cannot be included among the general acute-phase reactants, but there seems to be a close correlation between the serum level of antileukoprotease and an infectious disease of the respiratory tract, e.g. pneumonia (V). Whether this is the result of an increased secretion from the secretory cells or an increased leakage to the circulation is at present not known. An increased production and secretion would seem logical as acute inflammatory processes in the lung are accompanied by an increased release of granulocyte elastase (90,121).

The local balance between the proteases and their inhibitors is of importance for protection not only of the lung parenchyma but also of the mucous membranes (128). Thus, in middle ear secretions, there is a residual inhibitory capacity in serous otitis media, whereas the inhibitors are almost entirely consumed in acute otitis media (14). Purulent bronchial secretions usually show free proteolytic activity (90,120) whereas non-purulent secretions do not (71,102,125).

In an attempt to ascertain the local protective function of antileukoprotease, the complex formation of antileukoprotease and elastase was examined in purulent bronchial secretions (V). The total amount of antileukoprotease was recovered in complex with elastase in the sputa with free elastase activity. The elastase activity in these sputa originated from an excess of free granulocyte elastase. The sputa without elastase activity had some antileukoprotease in complex with elastase. In addition, large amounts of free antileukoprotease were found in these latter sputa (V). It has earlier been shown that antileukoprotease can block the deleterious effects of granulocyte

elastase on the ciliary function *in vitro* (128).

Several additional inhibitors have been described in bronchial secretions (39,41). One fragment of the inter- α -trypsin inhibitor, found in bronchial secretions, may inhibit elastase (21,57,60). These inhibitors, together with α_2 -macroglobulin, contribute, however, only to a minor part of the total inhibitory capacity of bronchial secretions.

The significance of antichymotrypsin as an inhibitor of cathepsin G is uncertain. The finding that cathepsin G may stimulate the rate of degradation of human lung elastin by granulocyte elastase gives perhaps antichymotrypsin an essential protective role (12).

The presence of antileukoprotease in complex with elastase in bronchial secretions in bronchitis indicates a local protective function of antileukoprotease. Furthermore, antileukoprotease may act as an inhibitor of elastase bound to α_2 -macroglobulin. These findings support the theory that antileukoprotease has a physiological function as a defence mechanism against the effects of granulocyte elastase, at least on the mucous membranes of the respiratory tract. The presence of antileukoprotease in serum indicates that the inhibitor reaches the interstitial tissues. This occurs more readily in inflammatory processes as judged from the ten-fold increased serum levels. Whether these findings also imply a role for antileukoprotease in the tissues as a defence against elastase remains to be proven.

SUMMARY AND CONCLUSIONS

Immunohistochemical examination of human tracheal and maxillary sinus mucosa revealed antileukoprotease in the serous parts of the seromucous glands. These results indicate that antileukoprotease has its production site in these glands and can be included among the secretory proteins.

Using the radioimmunoassay for antileukoprotease the serum concentration in healthy blood donors was found to be 126 ± 19 $\mu\text{g/l}$. Gel-filtration of sera showed that antileukoprotease was present in a free form and that addition of granulocyte elastase resulted in complex formation of antileukoprotease and elastase. This complex showed an altered and diminished antileukoprotease immunoreactivity. The antileukoprotease-elastase complex is stable *in vitro* and does not dissociate during 24 hours of incubation. The addition of the antileukoprotease-elastase complex to human serum results in a rapid dissociation. Antileukoprotease is recovered in a free form and elastase is found bound to α_1 -antitrypsin and to a small degree to α_2 -macroglobulin. The dissociation of the antileukoprotease-elastase complex in serum can probably be explained by the extremely high binding rate between elastase and α_1 -antitrypsin.

The α_2 -macroglobulin-bound elastase displays some elastase activity on low molecular weight substrate. The addition of antileukoprotease to complexes of α_2 -macroglobulin and elastase resulted in a decrease of the elastase activity. Thus, antileukoprotease is capable of inhibiting α_2 -macroglobulin-bound elastase at least *in vitro*. Elastase bound to α_2 -macroglobulin in a molar ratio of 1:1 is more susceptible to inhibition by antileukoprotease than when it is bound in a molar ratio of 2:1. The inhibition was, however, a slow process reaching equilibrium within hours, which might argue against a biological role for antileukoprotease as an inhibitor of elastase- α_2 -macroglobulin complexes.

Antileukoprotease as well as α_1 -antitrypsin are affected by cigarette smoke condensate *in vitro*, resulting in a changed immunoelectrophoretic pattern and a diminished inhibitory capacity of granulocyte elastase. Also, elastase shows a

decreased proteolytic activity after incubation with cigarette smoke condensate. The influence of cigarette smoking on the protease-antiprotease balance in the respiratory tract is complicated and it is not easily elucidated only by *in vitro* experiments.

The circulating levels of antileukoprotease, α_1 -antitrypsin and antichymotrypsin, are significantly increased in patients with pneumonia. The serum level decreased faster for antileukoprotease than for α_1 -antitrypsin and antichymotrypsin in these patients. Surgical trauma does not affect antileukoprotease as distinguished from the general acute-phase reactants. Gel-filtration of sera from patients with pneumonia revealed antileukoprotease in a free form despite the ten-fold increase of the antileukoprotease level.

In bronchial secretions antileukoprotease was recovered in complex with elastase. The sputa without elastase activity had an excess of free antileukoprotease, whereas in the sputa with elastase activity the total amount of antileukoprotease was in complex with elastase, i.e. the inhibitor was consumed.

The present investigation has shown that antileukoprotease can be located in the serous parts of the seromucous glands in the respiratory tract mucosa, where it probably has its production site. As a secretory protein, antileukoprotease is present in a free and active form in trace amounts in serum, in health as well as in disease. Any antileukoprotease-elastase complexes reaching the circulation are rapidly cleaved, the elastase being bound by the plasma protease inhibitors. These properties place antileukoprotease in the same group as other acid stable and low molecular weight inhibitors with established local function. Antileukoprotease does not react as a general acute-phase reactant, but the serum level may reflect an infectious respiratory disease, e.g. pneumonia. In purulent bronchial secretions antileukoprotease is present in complex, indicating a local protective function.

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DISTRIBUTION OF ANTILEUKOPROTEASE IN UPPER RESPIRATORY MUCOSA

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Human respiratory tract secretions contain enzyme inhibitors derived from plasma and a low molecular weight, acid-stable protease inhibitor, antileukoprotease. The distribution of antileukoprotease in normal upper respiratory tract mucosa has been studied using an immunohistologic technique. The inhibitor was localized to the serous parts of the submucosal glands of the maxillary sinus and the trachea but was not demonstrable in mucous glands and goblet cells. It is concluded that the antileukoprotease found in respiratory tract secretions is produced locally in the submucosal serous glands.

INTRODUCTION

Antileukoprotease is a potent inhibitor of human leukocyte elastase^{1,2} and chymotrypsin-like cationic proteins.^{2,3} It accounts for about 90% of the protease-inhibiting capacity of human bronchial secretions.⁴ The remainder of the inhibiting capacity is mainly accounted for by α_1 -antitrypsin and α_1 -anti-chymotrypsin.⁴ Antileukoprotease has not been found in plasma using an immunodiffusion technique.¹ However, using a new radioimmunoassay for the inhibitor, we have recently found antileukoprotease in human serum in a concentration of about 125 $\mu\text{g/l}$.⁵

Antileukoprotease was originally localized to mucosal and submucosal structures of the upper airways in our laboratory with an indirect immunoperoxidase technique.⁶ The results obtained did not allow detailed analyses as to cell localization of the inhibitor. The purpose of the present investigation was therefore to establish more exact localization for the inhibitor by applying recent improvements of an immunohistologic technique. The method basically utilizes immune complexes of peroxidase with rabbit antibody against peroxidase (PAP) according to Sternberger.⁷

METHODS AND MATERIALS

MATERIALS

Materials used in this study are as follows: 3,3-diaminobenzidine-tetrahydrochloride from BDH Chemicals Ltd, Poole, England; Pertex, from Histolab, Bethlehem Trading Ltd, Gothenburg, Sweden; swine-antirabbit IgG, PAP and normal swine serum from Dako Immunoglobulins, Copenhagen; Shiffs reagents from Merck, Darmstadt, Germany; and pepsin from Sigma Chemical Co, Stockholm. Monospecific rabbit antiserum against the low molecular weight inhibitor from bronchial secretions was

prepared as described earlier.¹ A sample of the antiserum was absorbed with antileukoprotease isolated as described earlier⁸ for use with the controls.

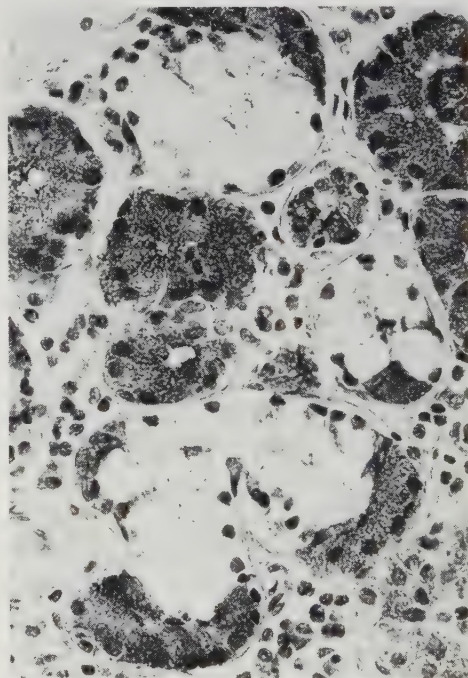


Fig. 1. Seromucous gland of tracheal mucosa. Positive (black) staining of the serous glands, semilunary surrounding the negative mucous glands. (Panchrome green filter, $\times 450$)

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ANTILEUKOPROTEASE IN RESPIRATORY MUCOSA

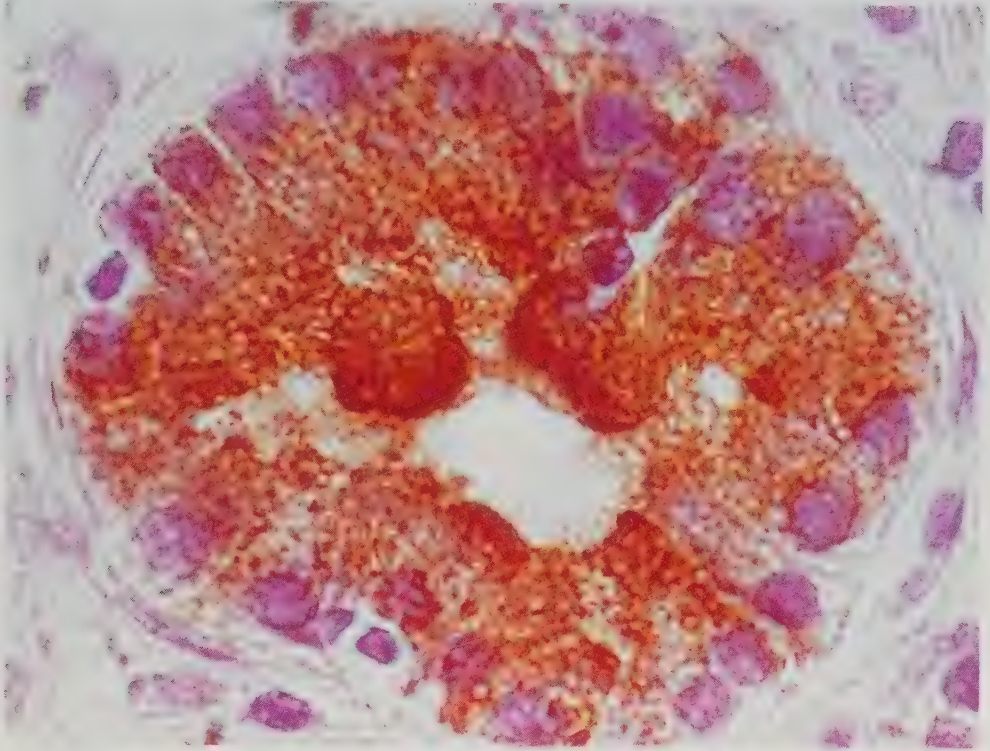


Fig. 2. Positive (brown) staining for antileukoprotease in serous gland cells of tracheal mucosa. (x 1250)

Tissue Sections. Human tracheal mucous membranes were taken at operation from three patients undergoing tracheotomy. Maxillary sinus mucosa was obtained from three patients undergoing surgical correction of facial deformities.

METHODS

The fixation and immunological staining procedures were adopted and slightly modified from earlier publications^{7,9,10} as follows:

1. Tracheal and maxillary sinus mucosa were fixed immediately in buffered formalin solution for more than 24 hours and then imbedded in histowax.
2. Sections were cut at 5 μ , mounted on glass slides, dewaxed and hydrated by washing for 2-3 minutes in xylene, ethanol 99.5%, and ethanol 96% and Tris-buffer successively. The Tris-buffer (0.05M Tris-HCl, pH 7.6 containing 0.15M NaCl) was used for all serum dilutions and washings. Washing was performed in three changes of the Tris-HCl buffer, each for 30 minutes.
3. Sections were incubated for 30 minutes at 37°C in a solution of pepsin (4 mg/ml in 0.01M HCl) to reduce background staining.¹⁰
4. Endogenous peroxidase activity was blocked by incubation with a 0.3% solution of hydrogen peroxide in methanol for 30 minutes.
5. To further reduce nonspecific background staining, sections were treated for 10 minutes with normal, nonimmune swine serum, diluted 1:20.⁹
6. Specific antiserum to antileukoprotease in seven different dilutions (1:20, 1:50, 1:100, 1:200, 1:500, 1:1000, 1:2000) was applied for 30 minutes. Controls were incubated with the same dilu-

tions of the specific antiserum, which had been previously absorbed to purified antileukoprotease.

7. Sections were treated with swine-antirabbit IgG, diluted 1:20, for 30 minutes.

8. Peroxidase-antiperoxidase (PAP) complex was then added for 30 minutes in a dilution of 1:20.

9. The sections were developed with diaminobenzidine (0.6 g/l) and hydrogen peroxide (0.01%) for 5-10 minutes.

10. After counterstaining with the PAS-method or hematoxylin, sections were dehydrated and mounted in Pertex. All steps were carried out at room temperature and in a humidified chamber.

RESULTS AND DISCUSSION

The high concentrations of antileukoprotease found in bronchial secretions⁴ suggested local production of the inhibitor. In a previous study using an immunoperoxidase method, we found positive staining of the seromucous glands of the tracheal mucosa and also of the ciliated epithelium indicating a mucosal production site of antileukoprotease.⁶ To localize the productive site more specifically, we combined a more sophisticated immunoperoxidase method with PAS staining. To reduce background staining, we incubated sections first in pepsin and then in swine serum, also higher dilutions of antiserum were used and the sections were washed more thoroughly. The most suitable dilutions of our

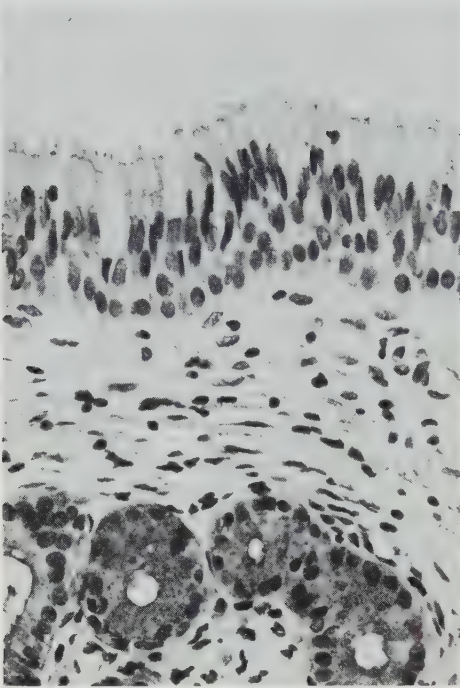


Fig. 3. Maxillary sinus mucosa. Positive (black) staining for antileukoprotease in serous gland cells. Ciliated epithelium with goblet cell negative. (Panchrome green filter, x 450)

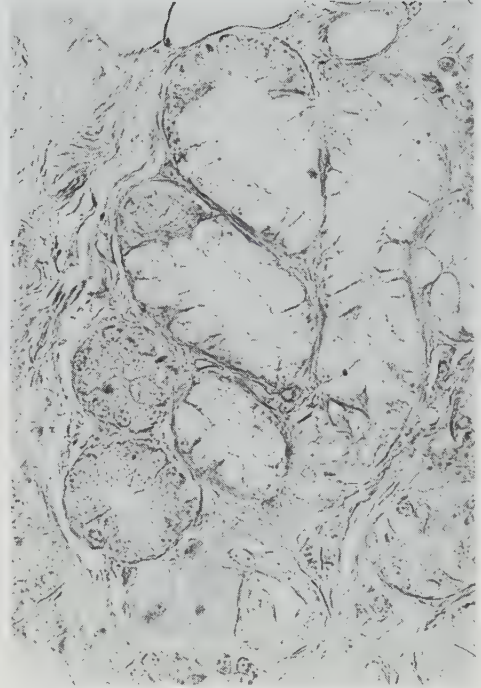


Fig. 4. Control. Serous and mucous gland cells of tracheal mucosa incubated with specific antiserum previously absorbed with antileukoprotease showing no positive staining. (Panchrome green filter, x 450)

antiserum were 1:1000-1:2000. Sections prepared with this method showed no background staining.

Morphological detail in all sections examined was excellent. Positive brown staining for antileukoprotease contrasted well with the blue hematoxylin or the pink PAS counterstains. Positive staining for antileukoprotease was confined to the serous parts of the seromucous glands of both tracheal (Figs. 1 and 2) and maxillary sinus mucosa (Fig. 3). The PAS-positive cells contained no demonstrable antileukoprotease nor did any cell of the ciliated epithelium (Fig. 3). In control sections handled in the same way but incubated with the specific antiserum previously adsorbed with antileukoprotease, the positive staining was completely abolished confirming the specificity of the reaction for antileukoprotease (Fig. 4). The present results strongly indicate that antileukoprotease found in bronchial secretions is

produced in the respiratory tract mucosa by the submucosal serous glands. This conclusion is supported by the absence of any demonstrable immunologic cross-reactivity with plasma proteins.⁵ Preliminary studies of the immunoreactive antileukoprotease found in serum show that this protein has a molecular weight of about 10,000, indicating a noncomplexed inhibitor.

It remains to be proven, although it seems most likely, that antileukoprotease found in serum is derived mainly from the respiratory tract. The same inhibitor appears to be produced in vesicula seminalis and in the uterine cervical mucosa. However, given the size differences between the respiratory and other sources, it seems likely that the respiratory tract is the main source of serum antileukoprotease.²

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A Radioimmunoassay for Measurement and Characterization of Human Antileukoprotease in Serum

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Summary: This report describes a radioimmunological method for the measurement of the protease inhibitor antileukoprotease in nanogram quantities. Antileukoprotease, previously found in bronchial secretions, has now also been found in serum using this radioimmunologic technique. In healthy blood donors the serum level was 126 $\mu\text{g/l}$.

The serum immunoreactive antileukoprotease was found in a free form and did not show any antigenic cross reaction with the normal plasma protease inhibitors. Further, it was also found to be active and to form a complex with active human granulocyte elastase.

Radioimmuntest zur Messung und Charakterisierung von menschlicher Antileukoprotease im Serum

Zusammenfassung: Es wird eine radioimmunologische Methode zur Messung des Proteaseinhibitors Antileukoprotease in ng-Mengen beschrieben. Mit dieser Methode wurde Antileukoprotease nun auch im Serum gefunden, nachdem ihr Vorkommen in Bronchialsekreten bereits früher bekannt war. Bei gesunden Blutspendern betrug der Se-

rumspiegel 126 $\mu\text{g/l}$. Die immunreaktive Serum-Antileukoprotease lag in freier Form vor und zeigte keine antigenen Kreuzreaktionen mit normalen Plasma-Protease-Inhibitoren. Sie war aktiv und bildete mit aktiver menschlicher Granulozyten-Elastase einen Komplex.

Key words: Cathepsin G inhibitors, elastase inhibitors, granulocyte elastase, protease inhibitors, radioimmunoassay.

Human bronchial secretions contain a low-molecular weight ($M_r \sim 10500$) protease inhibitor, antileukoprotease, which is a potent inhibitor of granulocyte elastase and chymotrypsin-like cationic proteins^[1–3]. It also appears to be present in the genital tract secretions of men and women^[4]. Antileukoprotease was found to account for 90% of the inhibiting capacity in bronchial lavage fluids^[5] and appeared to be important for the integrity of the mucus membranes in

the respiratory tractus^[6]. In previous studies radial immunodiffusion was used for localization and quantitation of antileukoprotease^[5]. This method is, however, not sensitive enough to detect antileukoprotease in serum.

The purpose of this study was to develop a method which would permit quantitation of antileukoprotease in nanogram quantities, thus allowing the protein to be analyzed in serum, as judged from other secretory proteins like trypsinogen^[7].

Materials and Methods

Special materials

Sephadex G-50 and G-75 are products of AB Pharmacia Fine Chemicals, Uppsala, Sweden. Bovine serum albumin was purchased from Miles Laboratories Ltd. Human granulocyte elastase is available in our laboratory^[8].

Antisera

Normal sheep serum and rabbit anti-sheep IgG antiserum are produced in our laboratory. Antileukoprotease and the IgG fraction of the antiserum were prepared as described by Ohlsson et al.^[2,9].

Human biological material

Serum samples were obtained from 60 healthy blood donors and from 10 patients 3–12 months following total hysterectomy. The blood was kept at +4 °C and serum was separated off through centrifugation within 24 h after the blood was drawn.

The samples were then frozen at -20 °C and thawed just before use.

Radioactive labelling of antileukoprotease

Five µg of antileukoprotease was labelled with 37 MBq of ¹²⁵I using the lactoperoxidase method of Thorell and Johansson, 1971^[10]. Average specific activity from several labellings was about 2600 MBq/mg. Separation was carried out on a Sephadex G-50 column (1.6 × 45 cm) using 0.01M Tris pH 7.4 plus 0.5M NaCl, 0.005M EDTA and 0.03nM bovine serum albumin. Fraction volumes were 2 ml and a 10-µl aliquot of each was counted in a well-type scintillation detector. The fraction containing the highest counts was divided into small aliquot portions and frozen at -70 °C.

Precipitation experiments

Precipitation experiments were performed with the tracer and antibodies against antileukoprotease (IgG fraction from antiserum against antileukoprotease). All dilutions were made with Tris/HCl buffer (0.01M Tris/HCl, 0.12M NaCl, 0.005M EDTA and 0.03nM bovine serum albumin, pH 7.4). A solution of the labelled antileukoprotease containing 1.75 ng/ml was prepared. 200 µl of the tracer solution was incubated with 200 µl of antileukoprotease IgG (1 mg/ml) diluted from 1:50–1:128000. 100 µl buffer was added and the solutions were mixed and incubated 20 to 24 h at +4 °C. Then 100 µl normal sheep serum (1:400) and 100 µl rabbit anti-sheep IgG serum (1:5) both diluted with the same buffer, together with 500 µl of the buffer, were added to the samples, mixed and incubated overnight at +4 °C. The reaction mixtures were then centrifuged at 2000 × g for 15 min. The supernatant was decanted and the radioactivity of the precipitate

measured. 82% of the radioactivity could be precipitated with an antiserum dilution of 1:8000, and 50% with the antiserum in a dilution of 1:90000.

Radioimmunological assay procedure

The previously described buffer was used for all dilutions. Standard solutions in concentrations from 0 to 1000 ng antileukoprotease/ml were prepared. Tracer was used at a concentration of 1.75 ng/ml, corresponding to about 50000 cpm/200 µl.

The antiserum was used in a dilution of 1:30000 yielding about 78% precipitation of the radioactivity, when no additional antileukoprotease was present.

For the assay, each tube contained 100 µl standard solution or 100 µl of a sample diluted 1:10 or 1:20 and also in each tube there was 200 µl antibody solution (1:30000) and 200 µl tracer. The tubes were thoroughly mixed and incubated overnight at +4 °C.

After this overnight incubation, 100 µl sheep serum (1:400), 100 µl rabbit anti-sheep IgG (1:5) and 500 µl of the buffer were added. The tubes were then agitated, incubated overnight at 4 °C, and thereafter centrifuged at 2000 × g for 15 min. The supernatant was decanted and the radioactivity of the pellet was measured. Both standards and samples were run in duplicate assay tubes.

Results

Properties of the method

Specificity. The standard dilution curve for antileukoprotease was compared with a dilution curve, based on serum samples from 6 healthy blood donors, and the curves in Fig. 1 represent the mean of the samples. All samples were run in duplicate assay tubes. The curves appear parallel indicating that the same immunoreactive substance is measured in the different dilution series (Fig. 1).

Sensitivity (the lowest amount of ligand distinguishable from zero). The sensitivity of the assay was 2 µg/l which was adequate to measure the antileukoprotease concentration in sampled sera from healthy blood donors.

Accuracy. The measure results had a coefficient of variation within assay of 5.96%.

Antileukoprotease in normal serum

Serum prepared from healthy blood donors contained 126 ng/ml ± 19.5 (n = 60). When the samples were redetermined after having been refrozen at -20 °C and thawed again, there were no significant changes in the concentrations. The recovery

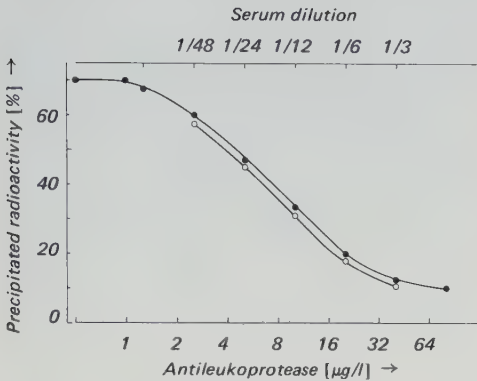


Fig. 1. Radioimmunoassay for antileukoprotease. Comparison between standard dilution curves of antileukoprotease in the dilution buffer (●—●) and mean of six normal sera (○—○).

of exogenous antileukoprotease added to normal serum is about 98% as shown in the table.

Gel filtration studies of normal serum

0.4-ml portions of three different normal serum samples were separated by gel filtration on a Sephadex G-75 column (0.9 × 60 cm) equilibrated with 0.01M Tris/HCl buffer containing 0.5M NaCl, pH 7.4.

Reaction mixtures containing 0.4 ml of the same serum samples and granulocyte elastase in slight excess of the elastase-binding capacity, as judged from crossed immunoelectrophoretic analyses^[11], were incubated for 15 min at 24 °C. They were then separated by gel filtration on the Sephadex

G-75 columns. Fractions were collected and measured with the radioimmunoassay for antileukoprotease. The peak of antileukoprotease eluted in a volume corresponding to the molecular weight of free antileukoprotease when no elastase was added to the sera. After the addition of granulocyte elastase to the sera, the immunoreactive antileukoprotease eluted in one considerably smaller peak (Fig. 2), with an elution volume corresponding to antileukoprotease-elastase complexes.

Studies on antileukoprotease in complex with elastase

Antileukoprotease was dissolved in a Tris/HCl buffer (0.05M Tris, 0.5M NaCl, pH 7.4) to give a

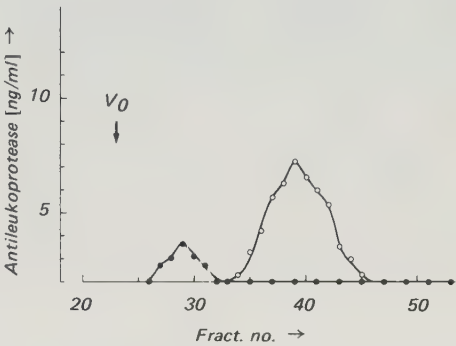


Fig. 2. Elution patterns of immunoreactive antileukoprotease after gel filtration on Sephadex G-75 of a serum sample before (○—○) and after (●—●) addition of granulocyte elastase.

Table. Recovery of antileukoprotease added to normal serum as measured by radioimmunoassay.

Exogenous antileukoprotease [ng/ml]	Radioimmunoassayable antileukoprotease [ng/ml]	Increase in antileukoprotease over endogenous material [ng/ml]	Recovery of exogenous antileukoprotease [%]
0	49	—	—
20	67	18	90
50	97	48	96
100	154	105	105
150	202	153	102

concentration of 125 $\mu\text{g/ml}$. To 400 μl of this antileukoprotease solution, 175 μg of leukocyte elastase in 100 μl of 0.001M HCl was added and the reaction mixture incubated for 10 min at 24 °C.

100 μl of an albumin solution (5 mg/ml, 0.15M NaCl) was added just before the reaction mixture was subjected to gel filtration on a G-75 Sephadex column (0.9 $\times$ 60 cm). Radioimmunoassay of the fractions for antileukoprotease showed a peak of immunoreactive antileukoprotease with an elution volume corresponding to the molecular weight of antileukoprotease-elastase complex. The fraction with the highest concentration of immunoreactive antileukoprotease was tested in a dilution series (1:10 – 1:10 240) using the radioimmunoassay after calculation of the true antileukoprotease content. The resultant curves were not parallel with the standard curve for antileukoprotease (Fig. 3). The immunoreactivity of antileukoprotease is clearly altered and diminished after complex formation with granulocyte elastase.

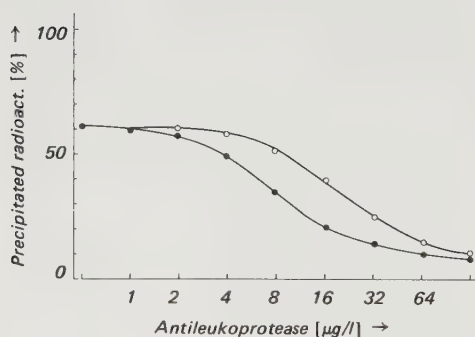


Fig. 3. Dilution curves for antileukoprotease (●—●) and the same amount of antileukoprotease after addition of granulocyte elastase in molar excess (○—○).

For details see results.

Antileukoprotease in serum from patients after hysterectomy

Serum samples from 10 patients, obtained about 3–12 months after operation contained antileukoprotease in a concentration of $140 \pm 29.2 \mu\text{g/l}$ serum.

Discussion

Human bronchial mucus secretion was originally shown by Hochstrasser et al.^[11] to contain an acid-stable, low-molecular inhibitor directed against granulocyte proteases. The inhibitor was later isolated^[2,9] and shown to have biochemical and anti-enzymatic properties identical to a low-molecular weight inhibitor purified from seminal plasma and cervical mucus^[4,12]. It seemed evident that all these mucus fluids contained the same inhibitory protein, a view which was supported by demonstrating an immunological precipitation of identity in Ouchterlony plates using the isolated inhibitors and their respective antisera^[13]. The inhibitor was designated antileukoprotease^[12] because its most striking property is its strong inhibition of granulocyte elastase^[2] and chymotrypsin-like cationic proteins^[3]. No cross reactivity was demonstrable between antileukoprotease and any plasma proteins, including inter- α -trypsin inhibitor, using immunoelectrophoretic or immunodiffusion techniques.

We anticipated, however, that antileukoprotease might be present in trace amounts in the serum as are secretory proteins from other glands, i.e. trypsinogen^[7]. This was found to be the case, but the concentration of antileukoprotease ($\sim 125 \mu\text{g/l}$) is about 5 times that of trypsinogen^[7]. The results of gel filtration analyses showed that antileukoprotease is present in a free form in serum and it has the ability to bind granulocyte elastase.

No other cross-reacting protein was demonstrable in the serum. After complexation of antileukoprotease with granulocyte elastase, some antigenic sites of the antileukoprotease molecule are still exposed and recognized by our antiserum, although the antileukoprotease immunoreactivity is considerably decreased. Radioimmunoassay measurements for antileukoprotease in reaction mixtures of normal serum with increasing amounts of antileukoprotease showed a recovery of about 98%.

The major source of the serum antileukoprotease is not self-evident, although it is likely to arise mainly from the respiratory tract mucosa, where we have recently localized antileukoprotease to the serous cells of the submucosal, seromucous glands with an immunohistologic technique^[14]. These glands are abundant in the respiratory tract,

especially in the trachea and larger bronchi and constitutes a much larger secretory potential than the cervical mucosa or the seminal vesicles. Furthermore, our preliminary quantitation of antileukoprotease in serum of a limited number of patients post hysterectomy indicates that the cervix does not contribute to a large extent to the serum level of antileukoprotease. We have, of course, not ruled out another as yet unidentified source of the inhibitor. Different organs are at the moment being screened in our laboratory with immunohistologic techniques to help resolve this problem.

The methods described in this paper should be useful in the future studies of the turnover and interactions of antileukoprotease in vivo both normally and in respiratory or genital tract diseases as well as in vitro experiments utilizing cell culture studies of respiratory or genital tract mucosa.

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STUDIES ON THE INTERACTION BETWEEN LEUKOCYTE ELASTASE, ANTILEUKO-
PROTEASE AND THE PLASMA PROTEASE INHIBITORS α_1 -ANTITRYPSIN AND
 α_2 -MACROGLOBULIN

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Abstract. The dominating inhibitor of leukocyte elastase in human respiratory tract secretions is a low molecular weight inhibitor, designated antileukoprotease. An equimolar antileukoprotease-elastase complex was produced and subjected to gel-filtration after differing time intervals and was found to be stable. On addition of human serum, however, elastase dissociated from antileukoprotease and formed a complex with α_1 -antitrypsin. A small amount of elastase was also found bound to α_2 -macroglobulin. Antileukoprotease was capable of inhibiting elastase bound to α_2 -macroglobulin. This inhibition was more complete and more rapid when the α_2 -macroglobulin-elastase complex was in a molar ratio of 1:1 than in a ratio of 1:2.

INTRODUCTION

Purulent secretions of the human respiratory tract contain several proteolytic enzymes such as elastase, neutral collagenolytic enzymes and cathepsin G (1,2). These enzymes are released extracellularly mainly from leukocytes. Leukocyte elastase is a potentially dangerous protease in the respiratory tract as it has been shown experimentally to destroy the ciliated respiratory tract mucosa and to be involved in the formation of emphysema (3,4). It is inhibited by the plasma protease inhibitors α_1 -antitrypsin and to a minor degree by α_2 -macroglobulin (1). The respiratory tract secretions contain also a low molecular weight acid stable inhibitor called antileukoprotease (5). Antileukoprotease is located in the serous parts of the seromucous glands in the respiratory tract (2). Antileukoprotease has turned out to be the dominating elastase inhibitor in the respiratory tract secretions of the upper airways, accounting for about 90 % of the total inhibitory capacity (6). The low molecular weight inhibitors present in human seminal plasma (HUSI-I) (7) and in uterine cervical secretions (CUSI) (8) are supposed to be identical with antileukoprotease (9).

The purpose of this study was to investigate the stability of an equimolar complex between antileukoprotease and elastase in serum and to elucidate the interaction of antileukoprotease with elastase bound to α_2 -macroglobulin.

MATERIAL

Sephadex G-25, G-75 and G-200 were purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden.

Agarose Seakem (batch 50013) was obtained from Marine Colloids Div. FMC Corporation, Rockland, USA.

Serum, freshly prepared, from healthy blood donors was used in the *in vitro* studies. The serum concentration of α_1 -antitrypsin was 1.3 mg/ml and of α_2 -macroglobulin 1.8 mg/ml.

Human leukocyte elastase, prepared as earlier described (10) was available at our laboratory.

N-succinyl-L-alanyl-L-alanyl-L-alanine-p-nitroanilide (Suc(Ala)₃pNan) was obtained from Protein Research Foundation, Peptide Institute Inc., Osaka, Japan.

Polyethylenglykol 6000 (PEG) was purchased from BDH, Chemicals Ltd, Poole, England.

Rabbit antisera against α_1 -antitrypsin and α_2 -macroglobulin were available at our laboratory.

Antileukoprotease and the antiserum against antileukoprotease were prepared as earlier described (5,11).

METHODS

Molecular weight. A molecular mass of 10.3 kDA for antileukoprotease and 33 kDA for elastase was used for all calculations (5,10). For α_2 -macroglobulin a molecular mass of 725 kDA was used (12).

Electrophoretic method. The electroimmunoassay of Laurell (13) was used for identifying α_1 -antitrypsin and α_2 -macroglobulin and their complexes with elastase. The agarose gel was made 0.8 % w/v.

Immunoassay of Mancini was used for identifying antileukoprotease (14). The agarose gel was made 0.8 %, containing 3 % w/v PEG (15).

Radioactive labelling of elastase and antileukoprotease was carried out following a lactoperoxidase method as described by Johansson and Thorell (16). To separate unbound iodide the labelled protein mixture was subjected to gel-filtration on a Sephadex G-25 column (1.6 x 20 cm) equilibrated with a Tris-HCl buffer, pH 7.6, containing 0.15 NaCl and 0.002 M CaCl₂.

The enzyme activity of elastase and the elastase inhibiting activity was measured with Suc(Ala)₃pNan as a substrate according to Bieth et al. (17).

Gel-filtration of the antileukoprotease-¹²⁵I-elastase complex was done on a Sephadex G-75 column (1.6 x 45 cm), equilibrated with 0.05 M Tris-HCl buffer, pH 7.4, containing 0.6 M NaCl. Reaction

mixtures of serum and antileukoprotease- ^{125}I -elastase or serum and ^{125}I -antileukoprotease-elastase complexes were analysed by gel-filtration on a Sephadex G-200 column (0.9 x 60 cm), equilibrated with 0.05 M Tris-HCl buffer, pH 7.6, containing 0.6 M NaCl and 0.002 M CaCl_2 .

Specific activity of reagents. Elastase was labelled with ^{125}I to a specific activity of about 96 $\mu\text{Ci}/\text{mg}$ using the lactoperoxidase method. Using the same method antileukoprotease was labelled with ^{125}I to a specific activity of about 122 $\mu\text{Ci}/\text{mg}$. The elastase and antileukoprotease retained their biological activity after labelling as shown by measurement of the elastase activity or elastase inhibiting capacity of the labelled protein after gel-filtration.

EXPERIMENTAL

1. Production of ^{125}I -elastase-antileukoprotease and ^{125}I -antileukoprotease-elastase complexes

The labelled leukocyte elastase was titrated with turkey ovomucoid as previously described (18). The elastase was then used to titrate solutions of the labelled and unlabelled antileukoprotease.

a. In a molar ratio of about 4:5, ^{125}I -labelled elastase was incubated with antileukoprotease for 10 min at room temperature to form ^{125}I -elastase-antileukoprotease complexes. The buffer used was 0.05 M Tris-HCl, pH 7.6, containing 0.002 M CaCl_2 and 0.15 M NaCl. Final volume of the reaction mixture was 300 μl .

b. In a molar ratio of about 5:4, ^{125}I -labelled antileukoprotease was incubated with elastase for 10 min at room temperature to form ^{125}I -antileukoprotease-elastase complexes, dissolved in the same Tris-HCl buffer to a final volume of 300 μl .

2. *Studies on the stability of the elastase-antileukoprotease complex*

a. Samples of the ^{125}I -elastase-antileukoprotease complex were subjected to gel-filtration on a Sephadex G-75 column after 10 min and after 24 h incubation at room temperature. For each of the collected fractions both radioactivity and elastase activity using $\text{Suc(Ala)}_3\text{pNan}$ as a substrate were determined. An immunoassay ad modum Mancini was performed for identification of antileukoprotease. The fractions containing the highest radioactivity were pooled, incubated for 10 min and 24 h, respectively, and samples of the pools were rechromatographed on the Sephadex G-75 column. The eluted fractions were analysed again as described above.

b. Fresh human serum was added to freshly prepared complexes of ^{125}I -elastase-antileukoprotease or ^{125}I -antileukoprotease-elastase in an amount giving a molar ratio of about 1:12 between the complex and serum α_2 -macroglobulin. The reaction mixtures were subjected to gel-filtration on Sephadex G-200 columns immediately and after 24 h incubation at room temperature. The distribution pattern of elastase and antileukoprotease in the chromatograms relative to α_1 -anti-trypsin and α_2 -macroglobulin and the changes of distribution with time were studied.

3. *Production of elastase- α_2 -macroglobulin complex*

Elastase in increasing amounts was added to constant volumes of freshly prepared human serum (100 μl). 0.2 M Tris-HCl buffer, pH 8.0, was added to give a final volume of 260 μl . After 15 min incubation $\text{Suc(Ala)}_3\text{pNan}$ (0.45 mg/ml) was added to each tube and thereafter the change in absorbance at 410 nm was followed up for 10 min. By extrapolation, the elastase binding capacity of 100 μl serum was estimated to about 95 μg of elastase.

On the basis of this calculation, 1.9 mg elastase was added to 2 ml human serum to prepare an elastase- α_2 -macroglobulin complex (in a molar ratio of about 2:1). The buffer used was 0.05 M Tris-HCl, pH 7.4, containing 0.5 M NaCl. The mixture was incubated for 10 min at room temperature

before gel-filtration on a Sephadex G-200 column (2.5 x 40 cm) was performed.

A similar procedure was carried out with 1.9 mg elastase added to 4 ml serum to prepare an elastase- α_2 -macroglobulin complex in a molar ratio of about 1:1. From both column runs, the fractions eluted in the molecular mass range of α_2 -macroglobulin were tested for elastase activity and those containing most activity were pooled.

4. *Inhibition of α_2 -macroglobulin-elastase complexes by antileukoprotease*

150 μ l and 300 μ l portions containing a constant amount of elastase- α_2 -macroglobulin complex, ratio 2:1 and 1:1, respectively, dissolved in 0.2 M Tris-HCl buffer, pH 8.0, were incubated with increasing amounts of antileukoprotease (0, 2.5, 5, 10, 20 μ g) to a final volume of 400 μ l. After 1 h, 4 h and 24 h incubation at room temperature, Suc(Ala)₃pNan was added and the change in absorbance at 410 nm was followed up for 10 min for detection of elastase activity.

RESULTS

Stability of 125 I-elastase-antileukoprotease complexes

On gel-filtration of the 125 I-elastase-antileukoprotease complex through a Sephadex G-75 column after 10 min and 24 h incubation, the radioactivity appeared as a single peak, which coincided with the size of the precipitates of the immunoassay for antileukoprotease. No elastase activity was detectable with Suc(Ala)₃pNan as a substrate. Rechromatography of the pooled complex-containing fractions after 24 h incubation at room temperature gave the same result (Fig. 1).

Stability of complexes between elastase and antileukoprotease in human serum

a. Gel-filtration of mixtures of serum and the 125 I-elastase-antileukoprotease complex after various times of incubation (immediately and after 24 h) showed that the main part of the radioactivity was confined to two peaks, corresponding to the

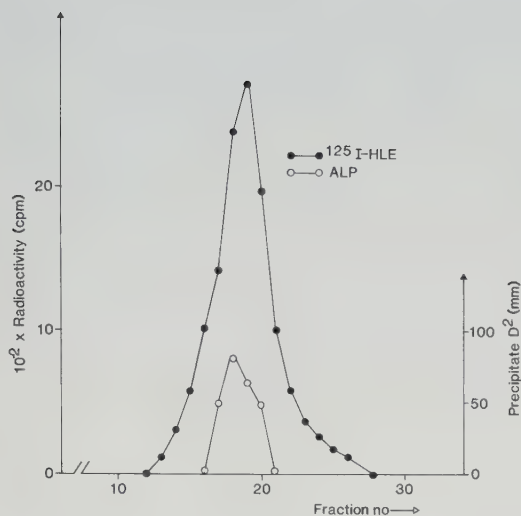


Fig. 1. Partition of radioactivity of ^{125}I -elastase (HLE) and immunoreactive antileukoprotease (ALP) among the fractions obtained by gel-filtration through a Sephadex G-75 column if the ^{125}I -elastase-antileukoprotease complex was rechromatographed.

fractions containing α_1 -antitrypsin and α_2 -macroglobulin. The pattern of precipitates obtained in the electroimmunoassay with α_1 -antitrypsin or α_2 -macroglobulin was close to the elution volume of the α_1 -antitrypsin-elastase or α_2 -macroglobulin-elastase complex. By immunoprecipitation it could be demonstrated that about 90 % of the radioactivity was associated with α_1 -antitrypsin and only 10 % with α_2 -macroglobulin when the mixture was subjected immediately to gel-filtration. After 24 h of incubation that ratio changed to 75 % and 25 % for α_1 -antitrypsin and α_2 -macroglobulin, respectively. The prolonged slope of the ^{125}I -elastase curve in Fig. 2b is suggested to originate from biologically inactive elastase, as there is no corresponding ^{125}I -antileukoprotease peak in Fig. 3b. Antileukoprotease was located with immunoassay ad modum Mancini, to the fractions corresponding to the molecular weight of free antileukoprotease. Some elastase activity, tested with $\text{Suc(Ala)}_3\text{pNan}$ as a substrate, was observed in the fractions containing α_2 -macroglobulin (Fig. 2a and b).

b. After gel-filtration of mixtures of serum and ^{125}I -antileukoprotease-elastase complex, more than 80 % of the radioactivity eluted in a volume corresponding to the molecular weight of free

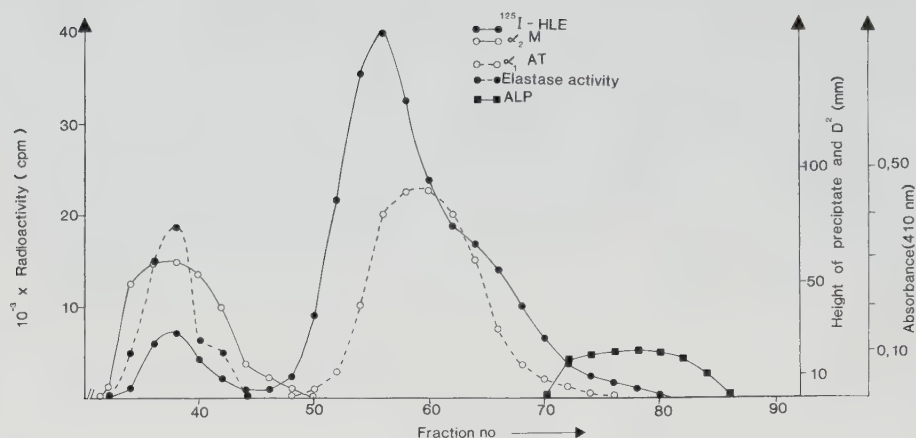


Fig. 2. Distribution of radioactivity, immunoreactive α_1 -anti-trypsin (α_1 -AT) and α_2 -macroglobulin (α_2 -M) and elastase activity among the fractions obtained by gel-filtration through a Sephadex G-200 column of a mixture of human serum and freshly prepared ^{125}I -elastase-antileukoprotease complex.

a. The mixture subjected immediately to gel-filtration.

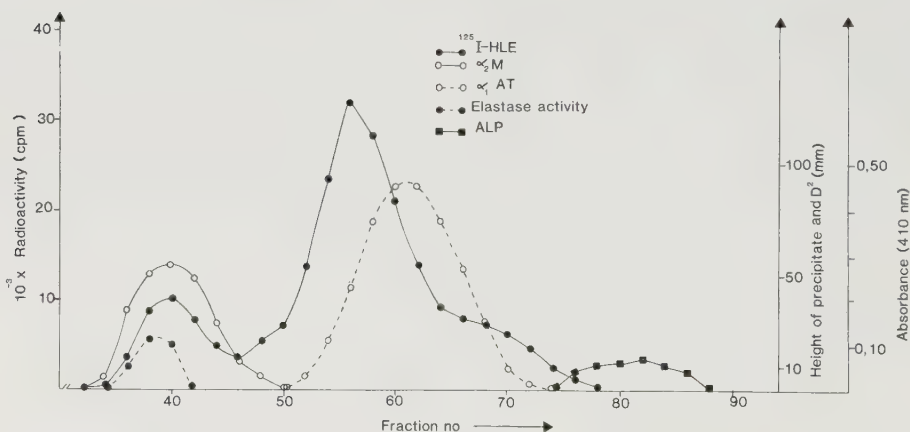


Fig. 2. Distribution of radioactivity, immunoreactive α_1 -anti-trypsin (α_1 -AT) and α_2 -macroglobulin (α_2 -M) and elastase activity among the fractions obtained by gel-filtration through a Sephadex G-200 column of a mixture of human serum and freshly prepared ^{125}I -elastase-antileukoprotease complex.

b. The mixture subjected to gel-filtration after 24 h incubation at room temperature.

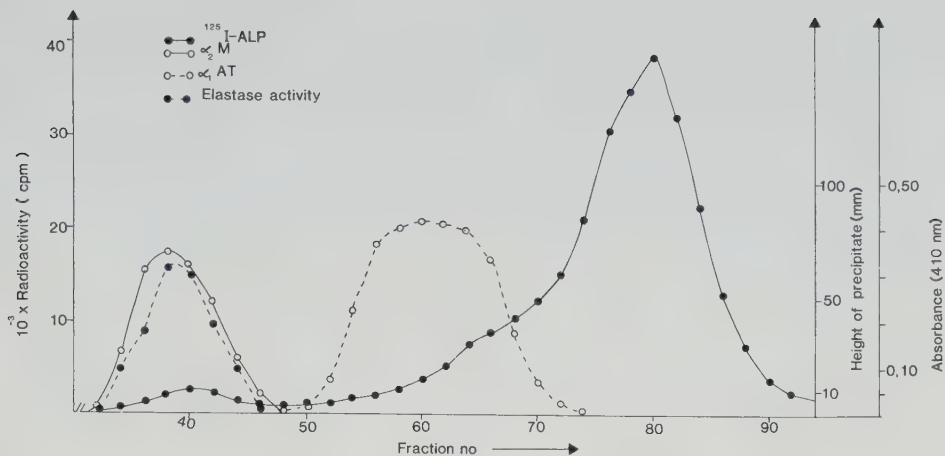


Fig. 3. Distribution of radioactivity, immunoreactive α_1 -anti-trypsin (α_1 -AT) and α_2 -macroglobulin (α_2 -M) and elastase activity among the fractions obtained by gel-filtration through a Sephadex G-200 column of a mixture of human serum and freshly prepared ^{125}I -antileukoprotease-elastase complex.
a. The mixture subjected immediately to gel-filtration.

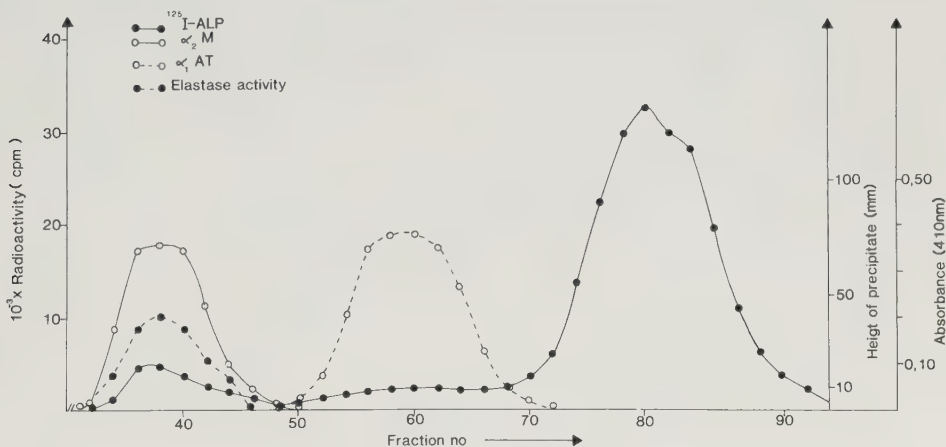


Fig. 3. Distribution of radioactivity, immunoreactive α_1 -anti-trypsin (α_1 -AT) and α_2 -macroglobulin (α_2 -M) and elastase activity among the fractions obtained by gel-filtration through a Sephadex G-200 column of a mixture of human serum and freshly prepared ^{125}I -antileukoprotease-elastase complex.
b. The mixture subjected to gel-filtration after 24 h incubation at room temperature.

antileukoprotease. About 4 % of the radioactivity was recovered in the α_2 -macroglobulin peak. Some radioactivity eluted also in a volume corresponding to the antileukoprotease-elastase complex. The mixture of serum and complex was preincubated for 24 h; ^{125}I -antileukoprotease was then recovered only in peaks corresponding to free antileukoprotease and α_2 -macroglobulin, indicating complete dissociation of the antileukoprotease-elastase complex during preincubation. Immunoassays performed for identification of α_2 -macroglobulin and α_1 -antitrypsin showed that the pattern of the precipitates coincided with the elution volume of the α_2 -macroglobulin-elastase complex and the α_1 -antitrypsin-elastase complex, respectively. In the fractions containing α_2 -macroglobulin, some elastase activity was found (Fig. 3a and b).

Inhibition of α_2 -macroglobulin-bound elastase by antileukoprotease

α_2 -Macroglobulin-elastase complexes (in molar ratios of 1:1 and 1:2) were incubated for 1 h, 4 h and 24 h with increasing amounts of antileukoprotease (Fig. 4). The inhibition of elastase

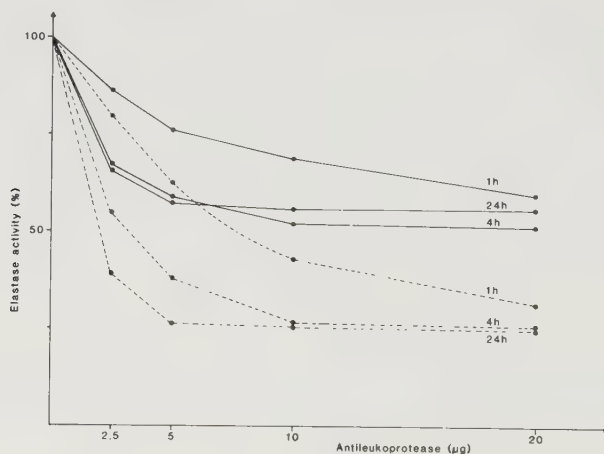


Fig. 4. Inhibition of α_2 -macroglobulin-bound elastase by increasing amounts of antileukoprotease (ALP) and after 1 h, 4 h and 24 h of incubation at room temperature. Ratio elastase to α_2 -macroglobulin (HLE: α_2 M) = 1:1 (---) and 2:1 (—). The amount of elastase tested in the aliquot was identical for the 1:1 and 2:1 elastase- α_2 -macroglobulin complexes.

activity was tested with $\text{Suc(Ala)}_3\text{pNan}$ as a substrate. The rate and degree of inhibition by antileukoprotease is lower when α_2 -macroglobulin is saturated by elastase than when the enzyme is present in less than a two-fold molar excess. By prolonging the incubation time the final degree of elastase inhibition is reached with decreasing quantities of added antileukoprotease.

DISCUSSION

The dominating protease inhibitors of the human respiratory tract, antileukoprotease and α_1 -antitrypsin, are the major inhibitors of leukocyte elastase (4). Dissociation of the antileukoprotease-elastase complex is possible by acid treatment (11) or by the action of α_1 -antitrypsin (18). Antileukoprotease dissociated from elastase is still active as an inhibitor (4), while α_1 -antitrypsin has lost its inhibiting capacity when it once has been bound to elastase (19,20). α_1 -Antitrypsin binds six times faster to elastase than does antileukoprotease, but the K_{ass} value for the antileukoprotease-elastase association is nevertheless among the highest ever reported for a protease-inhibitor system. Furthermore the K_i value of the elastase-antileukoprotease complex is $1.2 \cdot 10^{-11}$ (18). These data indicate that antileukoprotease forms a very stable complex with elastase. Our results support these observations by showing that an equimolar elastase-antileukoprotease complex is completely stable for at least 24 h. The addition of serum caused, however, a rapid dissociation of the complex. The main part of the elastase thus released was bound by α_1 -antitrypsin and only a smaller amount was found in the fractions containing α_2 -macroglobulin. Prolonged incubation increased, however, the α_2 -macroglobulin-bound proportion of elastase, possibly indicating a transfer of elastase initially bound by α_1 -antitrypsin.

The data obtained for antileukoprotease may be compared with those of another low molecular acid stable inhibitor, the pancreatic secretory trypsin inhibitor (PSTI) (21), which is a rather potent inhibitor of trypsin. Equimolar human PSTI-trypsin complexes dissociate slowly, but on addition of human serum to the complexes there is an immediate dissociation of the PSTI-trypsin complexes (21).

After addition of human serum to the antileukoprotease-elastase complex a major part of the antileukoprotease was obtained in a free form, but a small amount of the low molecular weight inhibitor was recovered in the elastase- α_2 -macroglobulin complex. This indicates that also molecules of a molecular mass of 10 kDA can gain access to α_2 -macroglobulin-bound proteases as compared with the earlier demonstration of an inhibition of enzymes bound to α_2 -macroglobulin by inhibitors in the region of 7 kDA like PSTI (21) and aprotinin (22).

Direct titration experiments showed, however, that the rate of inhibition of the α_2 -macroglobulin-elastase complex by antileukoprotease was very low compared with that of free elastase. Furthermore, very high concentrations of antileukoprotease were needed to reach appreciable inhibition. These data argue against any biological significance for antileukoprotease as an inhibitor of α_2 -macroglobulin-bound elastase. Such complexes have a half-life in the circulation of only 10 min (23) and are also rapidly phagocytised by alveolar macrophages *in vitro* ($T_{1/2} \sim 15$ min) (24).

The results concerning the interaction of antileukoprotease with α_2 -macroglobulin-bound elastase coincide with those of Bieth et al. (25) obtained in studies on α_2 -macroglobulin-bound trypsin inhibited by soybean trypsin inhibitor (STI). They found that the inhibition of α_2 -macroglobulin-bound trypsin in a ratio of 1:1 was more susceptible to inhibition by STI than the 2:1 complex and suggest a sterical hindrance to the reaction of STI with the second trypsin molecule.

Our results thus indicate that antileukoprotease functions primarily as an important inhibitor of granulocyte elastase in areas to which the plasma protease inhibitors have little or no access, like in the respiratory tract mucosa and secretions.

ACKNOWLEDGEMENTS

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The effect of cigarette smoke condensate on α_1 -antitrypsin, antileukoprotease and granulocyte elastase

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Abstract. Unfractionated cigarette smoke condensate was found to affect α_1 -antitrypsin and antileukoprotease, which are the dominating enzyme inhibitors in the respiratory tract. The electrophoretic and crossed immunoelectrophoretic precipitate patterns of these inhibitors were grossly altered and their inhibiting capacity reduced in both a dose and time dependent way. Other plasma proteins were also affected by cigarette smoke condensate, which in high concentrations caused precipitation of most serum proteins.

Human granulocyte elastase was reduced in functional activity by similar concentrations of the smoke condensate as the inhibitors. Although the elastolytic activity was markedly reduced, the esterolytic activity was reduced to a lesser degree.

Key words. α_1 -Antitrypsin, α -globulins, elastase, leucocytes, smoking, tobacco, trypsin inhibitor.

Introduction

Lung tissue destruction and pulmonary emphysema seem to be related to the effect of uninhibited proteolytic enzymes. In animals, aerosols of proteolytic enzymes have caused emphysematous lesions [1–3]. The early development of emphysema in persons with a genetic deficiency of the protease inhibitor α_1 -antitrypsin has been attributed to the lack of inhibition of granulocyte or macrophage elastase [4–6]. Another risk factor associated with the development of emphysema in man is cigarette smoking [7]. Recently cigarette smoke condensate has been shown to suppress the elastase inhibiting capacity of serum and bronchopulmonary secretions [8, 9].

The purpose of the present paper was to further elucidate the observation by Janoff [8] that cigarette smoke condensate suppresses the elastase inhibition of human α_1 -antitrypsin. We have utilized inhibition studies and analyses of the electrophoretic and crossed immunoelectrophoretic patterns of α_1 -antitrypsin

before and after exposure to smoke condensate. In addition, we studied the effect of smoke condensate on elastase and antileukoprotease, the dominating elastase inhibitor in bronchial secretions [10, 11].

Material and Methods

Special materials. DMSO (dimethylsulphoxide) was obtained from Sigma Chemical Co., St Louis, U.S.A., agarose for gel electrophoresis was obtained from Miles Seravac, Maidenhead, England.

Sephadex G-25 was a product of Pharmacia Fine Chemicals Inc., Uppsala, Sweden. Elastin from Bovine Ligamentum Nuchae was a product of Washington Biochemical Corporation, Freehold, N.J., U.S.A.

Suc(Ala)₃-pNA was obtained from Peptide Institute Protein Research Foundation, Osaka, Japan.

Human serum was obtained from a serum pool of blood donors. Purified human α_1 -antitrypsin was available at the laboratory. Antileukoprotease [12, 13] and granulocyte elastase [14] were isolated as previously described.

Antisera. Specific rabbit antiserum against α_1 -antitrypsin and antileukoprotease is available in our laboratory [13].

Electrophoretic methods. Standard techniques were used for agarose gel electrophoresis [15], electroimmunassay [16], crossed immunoelectrophoresis [17], and immunoelectrophoresis [18].

Enzyme activity and inhibition assays. The elastolytic activity of elastase was estimated from its digestion of elastin using the elastin agarose plate technique described earlier [14]. The elastase esterolytic activity was measured spectrophotometrically at 37°C using Suc(Ala)₃-pNA as substrate according to Bieth *et al.* [19].

The procedure used for the elastase inhibition studies with Suc(Ala)₃-pNA as substrate [19] is representative for the photometric tests and inhibition studies. 5 μ g granulocyte elastase, dissolved in HCl (1 mmol/l), was incubated with increasing amounts of α_1 -antitrypsin or antileukoprotease in Tris (0.2 mol/l

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adjusted to pH 7.4 by addition of 1 mol HCl, containing NaCl (150 mmol/l). The final volume was adjusted to 0.5 ml with the Tris-HCl buffer. Incubation took place for a minimum of 15 min at 37°C, and the reaction started by the addition of 1 ml of the substrate solution (1.35 mg Suc(Ala)₃-pNA/ml).

8.5 µg of our α_1 -antitrypsin preparation was found to completely inhibit 5 µg of elastase. In the following experiments the inhibitory effect of 8.5 µg α_1 -antitrypsin, with and without preincubation with varying amounts of smoke condensate solution on 5 µg elastase, was studied.

3 µg of antileukoprotease caused complete inhibition of 5 µg of elastase and was titrated in the same way as α_1 -antitrypsin.

Protein concentration in fractions after gel filtration was measured according to the method of Lowry [20] using the isolated corresponding proteins as standards.

Experimental procedure

Cigarette smoke condensates were prepared at the research laboratory of the Swedish Tobacco Company as recently described [21] and stored at -70°C. 20% weight volume (w/v) solutions of smoke condensate in dimethylsulphoxide (DMSO) was prepared immediately before use. The pH of a mixture of 100 µl of smoke condensate 20% (w/v) in DMSO and 100 µl of the Tris-HCl buffer was 7.4.

I. *Human serum* diluted 1:5 with saline (50 µl), was mixed with the Tris-HCl buffer so that the volume of the final reaction mixture would be 250 µl after the addition of increasing amounts of smoke condensate in DMSO 20% (w/v) (0–100 µl). Serum mixed with DMSO (100 µl) without smoke condensate was used as a control. Reaction mixtures were incubated for 18 h at 37°C and then analysed using agarose gel electrophoresis as described above.

II. *Human α_1 -antitrypsin* (1 mg) dissolved in saline was mixed with the Tris-HCl buffer, so that the volume of the final reaction mixture would be 250 µl after the addition of 20% (w/v) smoke condensate in DMSO (0–50 µl). Reaction mixtures were incubated at 37°C for 5 min, 1, 3, 6 and 18 h. The low molecular weight smoke condensate products were then separated off by gel filtration on identical Sephadex G-25 columns (1.5 × 6 cm) produced by Pharmacia Fine Chemicals Inc., equilibrated with the Tris-HCl buffer. The protein peak containing the smoke treated inhibitor was identified using electroimmunoassay, recovered, analysed by agarose gel electrophoresis, crossed immunoelectrophoresis, and used for inhibition studies.

In another study, 1 mg (50 µl) α_1 -antitrypsin was mixed with 190 µl of the Tris-HCl buffer and 10 µl of the smoke condensate solution. This mixture was then incubated for 2, 6, 12 and 18 h at 37°C. These samples were then subjected to gel filtration on the Sephadex G-25 columns (1.5 × 6 cm), and the protein containing

fractions pooled and analysed for elastase inhibition.

Control samples of α_1 -antitrypsin mixed with appropriate amounts of DMSO, but lacking smoke condensate, were treated in an identical manner.

III. *Human antileukoprotease* (0.5 mg) dissolved in saline was mixed with the Tris-HCl buffer, so that the final reaction mixture would be 125 µl with the addition of increasing amounts of 20% (w/v) smoke condensate in DMSO (0–25 µl). Antileukoprotease mixed with DMSO (25 µl) without smoke condensate was used as a control. The reaction mixtures were incubated at 37°C for 18 h before gel filtration on Sephadex G-25 as described above for α_1 -antitrypsin. The protein peaks were analysed by immunoelectrophoresis and also used in inhibition studies.

The inhibitory effect of untreated α_1 -antitrypsin and anti-leukoprotease, respectively, was used as reference.

IV. *Granulocyte elastase* (20 µg) in HCl (1 mmol/l; 2 mg/ml) was mixed with the Tris-HCl buffer, so that the final volume of the reaction mixture would be 100 µl after the addition of increasing amounts of 20% (w/v) smoke condensate in DMSO solution (0–25 µl). 10 µl samples were immediately analysed for elastolytic activity using the elastin agarose plate technique mentioned above. The plates were incubated at 37°C for 18 h, after which the diameters² of the elastolytic areas were measured and plotted.

Granulocyte elastase 20 µg in HCl (1 mmol/10 µl) was mixed with 80 µl of the Tris-HCl buffer. Smoke condensate in DMSO 20% (w/v) (10 µl) was added to each of these reaction mixtures and they were then incubated for 5, 25, 60, 120 and 180 min at 37°C. Granulocyte elastase mixed with DMSO alone was used as a reference. The low molecular weight smoke condensate products were then separated off by gel filtration on Sephadex G-25 as described above. The enzyme containing fractions were pooled and analysed for elastin and Suc(Ala)₃-pNA degrading activity as mentioned above. Final pH of all the reaction mixtures, I–IV, was 7.4.

No precipitation of any of the proteins in experiments II–IV occurred during incubation with DMSO smoke condensate or DMSO alone.

Results

The addition of smoke condensate in increasing amounts to human serum caused a progressive distortion of the electrophoretic pattern on agarose gel electrophoresis (Fig. 1). Albumin and α_1 -antitrypsin showed increased electrophoretic mobility rates at low concentrations of smoke condensate. With increasing amounts, the α_1 -antitrypsin disappeared. Other protein bands became progressively more diffuse, and with the highest concentration of smoke condensate added, only albumin was evident. This albumin band was wide, indicating electrophoretic heterogeneity.

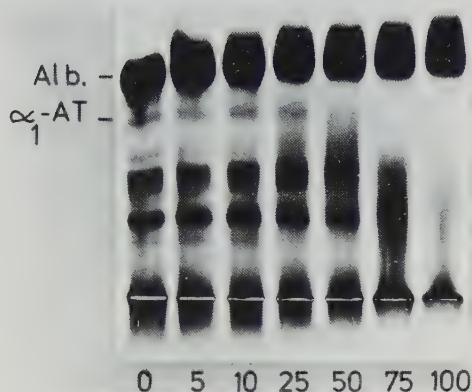


Figure 1. Agarose gel electrophoresis of normal human serum after addition of increasing amounts (0–100 μ l) of the DMSO solution of smoke condensate (20% w/v), and incubation for 18 h at 37°C. The serum sample without any smoke condensate solution added (0 μ l) was mixed with DMSO alone and used as a reference. All samples were run in the same gel.

Protein precipitates were found along the edges of the slit.

The progressive electrophoretic changes of α_1 -antitrypsin were made more obvious by the addition of smoke condensate solution to pure α_1 -antitrypsin. Increased mobility and pronounced band widening were readily apparent (Fig. 2). Further analysis of the reaction mixtures using crossed immunoelectrophoresis demonstrated a new α_1 -antitrypsin component, which gave a faint precipitate (Fig. 3). This new component had an electrophoretic mobility which increased directly as the amount of smoke condensate in the reaction mixture. A barely visible precipitate was seen in the β -region in some gels, further accentuating the molecular heterogeneity of α_1 -antitrypsin induced by the smoke condensate.

At the same time the original α_1 -antitrypsin component showed an increasing electrophoretic mobility rate, and the precipitate became progressively smaller with changed morphology (Fig. 3).

Immunoelectrophoresis of antileukoprotease after the addition of increasing amounts of smoke condensate showed progressive changes (Fig. 4). The most obvious change was a decreased cationic mobility.

Smoke condensate suppressed the inhibiting capacity of α_1 -antitrypsin against granulocyte elastase in a time and dose dependent way (Figs. 5 and 6). The addition of increasing amounts of smoke condensate

decreased the relative inhibitory capacity to approximately 10% (Fig. 5). Incubation of α_1 -antitrypsin with a constant amount of smoke condensate caused a time dependent decrease of the relative inhibitory capacity (Fig. 6).

The inhibitory function of antileukoprotease was also found to be affected by smoke condensate (Fig. 7). The inhibiting capacity against granulocyte elastase was suppressed by smoke condensate in a dose dependent way to about 25% of the control with the amounts of condensate tested.

Incubation of α_1 -antitrypsin or antileukoprotease with DMSO alone caused no measurable decrease in their inhibiting capacity.

Granulocyte elastase was tested for its elastolytic activity after the addition of increasing amounts of smoke condensate (Fig. 8). The elastin degrading activity was decreased to zero by increasing amounts of smoke condensate in a dose dependent fashion.

Granulocyte elastase mixed with 10 μ l of the DMSO-smoke condensate (20% w/v) was analysed following varying times of incubation (Fig. 9). Both the elastolytic and esterolytic activity of elastase were decreased following prolonged incubation. The decrease in enzymatic activity was more obvious for the elastolytic component. Elastase incubated with DMSO alone lost only about 10% of its enzymatic activity (Fig. 9).

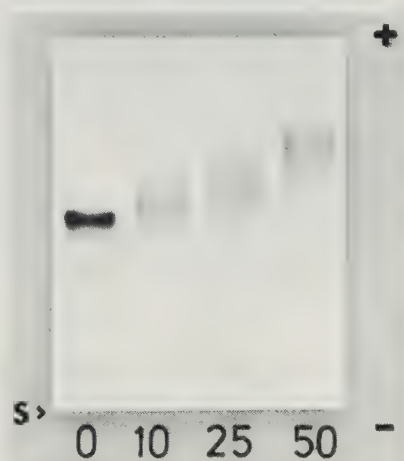


Figure 2. Agarose gel electrophoresis in the same plate of pure human α_1 -antitrypsin after addition of increasing amounts (0–50 μ l) of the DMSO solution of smoke condensate (20% w/v) and incubation for 18 h at 37°C. α_1 -Antitrypsin without smoke condensate solution added (0 μ l) was mixed with DMSO and used as a reference. Before agarose gel electrophoresis, the reaction mixtures were subjected to gel filtration on Sephadex G-25. S = site of origin

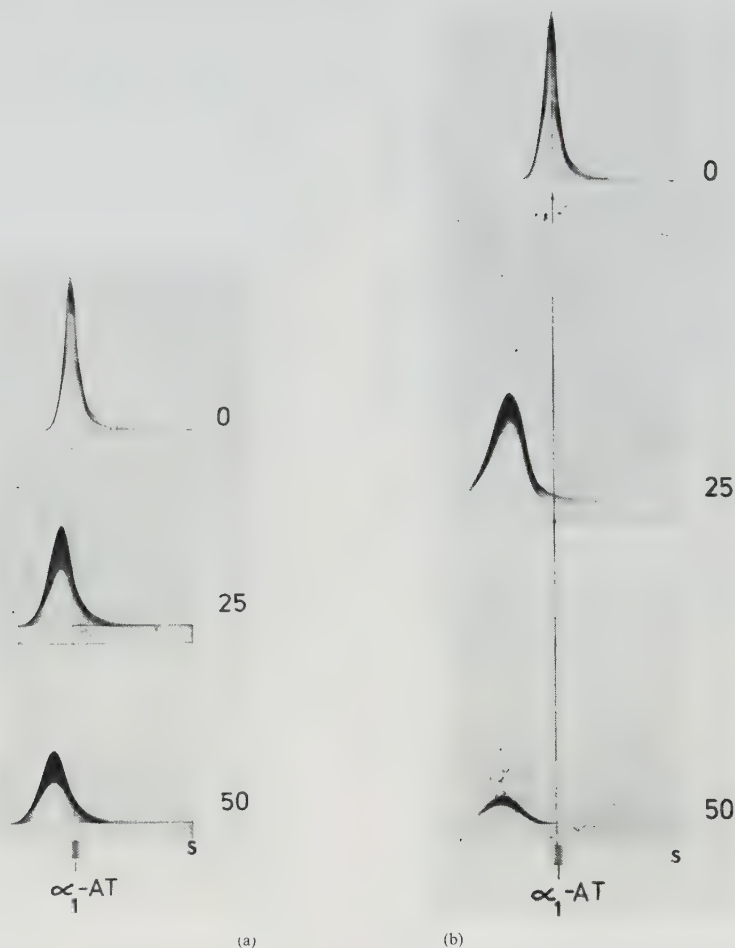


Figure 3. Crossed immunoelectrophoresis (in the same plate) of pure human α_1 -antitrypsin after addition of increasing amounts (0–50 μ l) of the DMSO solution of smoke condensate (20% w/v) and incubation for (a) 1 h and (b) 18 h at 37°C. α_1 -Antitrypsin without any smoke condensate solution added (0 μ l) was mixed with DMSO alone as a reference. The agarose gel contained antiserum against α_1 -antitrypsin. Before crossed immunoelectrophoresis, the reaction mixtures were subjected to gel filtration on Sephadex G-25. S=site of origin in first dimension.

Discussion

α_1 -Antitrypsin deficiency accounts for only a small fraction of persons with emphysema, whereas cigarette smoking is recognized as the major risk factor in most emphysema patients [7–9]. Thus the interest in Janoff's demonstration that cigarette smoke condensate suppressed the elastase inhibiting capacity of human serum, α_1 -antitrypsin, and bronchopulmonary lavage fluid [8]. We confirm Janoff's observations using a similar type of smoke condensate dissolved in DMSO. We have, however, also noted some differences, prob-

ably due to the use of different methods, which allowed more quantitative analyses.

It was evident from the agarose gel electrophoretic analyses that smoke condensates have a dramatic effect on most serum proteins, causing precipitation of many of them, when applied in large amounts. Less drastic amounts of smoke condensates, comparable to those used by Janoff, caused pronounced time and dose dependent changes of α_1 -antitrypsin, as evidenced by changes of band morphology on agarose gel electro-

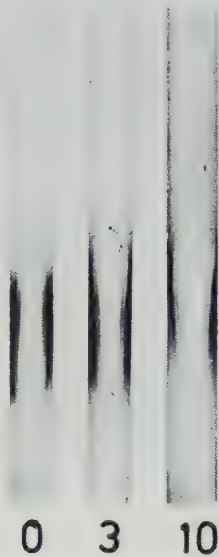


Figure 4. Immunelectrophoresis in one agarose gel plate of antileukoprotease after addition of increasing amounts (0–10 μ l) of the DMSO solution of smoke condensate (20% w/v) and incubation for 18 h at 37°C. Antileukoprotease without smoke condensate solution added (0 μ l) mixed with DMSO was used as a reference. Antiserum against antileukoprotease was used. Before immunelectrophoresis, the reaction mixtures were subjected to gel filtration on Sephadex G-25.

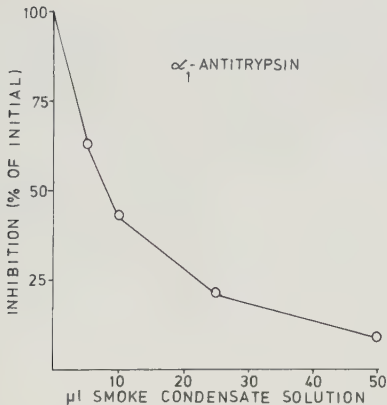


Figure 5. The inhibitory capacity of pure human α_1 -antitrypsin was tested after the addition of increasing amounts (0–50 μ l) of DMSO smoke condensate (20% w/v) solution and incubation for 18 h at 37°C. Controls were incubated with DMSO alone. Following incubation, the reaction mixtures were individually subjected to gel filtration on Sephadex G-25. 8.5 μ g of the eluted protein peak was incubated with 5 μ g elastase and the esterolytic activity measured. Incubation with DMSO caused no decrease in inhibiting capacity of α_1 -antitrypsin.

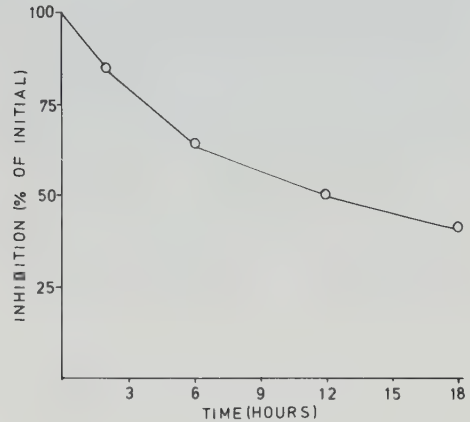


Figure 6. The inhibitory capacity of pure human α_1 -antitrypsin tested after addition of the DMSO smoke condensate (20% w/v) solution (10 μ l) and increasing time of incubation (0–18 h) at 37°C. (For procedure see Methods and legend to Fig. 5.) The α_1 -antitrypsin in the DMSO controls showed no decrease in inhibiting activity.

phoresis, and of precipitate patterns on crossed immunoelectrophoresis showing α_1 -antitrypsin components with varying immunoreactivity. In addition, α_1 -antitrypsin and also antileukoprotease became more anionic and less cationic, respectively. Thus, in contrast to Janoff [8, 9], we found evidence of drastic changes in the α_1 -antitrypsin molecule accompanying the loss of its functional effectiveness after exposure to components with denaturing effects in the cigarette smoke condensate.

The inhibition experiments similarly show a time and dose dependent suppression of the inhibiting capacity of α_1 -antitrypsin as well as antileukoprotease. The effect on antileukoprotease is especially interesting, as this inhibitor—which seems to be produced locally in the mucosa of the upper airways [22]—accounts for about 90% of the elastase inhibiting capacity in normal bronchial lavage fluids [10].

To our surprise, and in contrast to Janoff [8, 9], we found that the smoke condensates also suppressed the elastolytic as well as the esterolytic activity of granulocyte elastase. Judging from our present results, elastase seems to be at least as sensitive to cigarette smoke as the inhibitors. It would, however, be a gross oversimplification to draw the conclusion that the suppression of the inhibitory capacity of α_1 -antitrypsin and antileukoprotease by smoke is of little pathophysiological significance, since the enzymatic activity of granulocyte elastase is likewise suppressed. The present results do not allow any conclusions to be drawn as to the influence of cigarette smoking on the protease-antiprotease balance in the lung *in vivo*. Direct studies of this balance in bronchial secretions from smokers with and without airway disease will

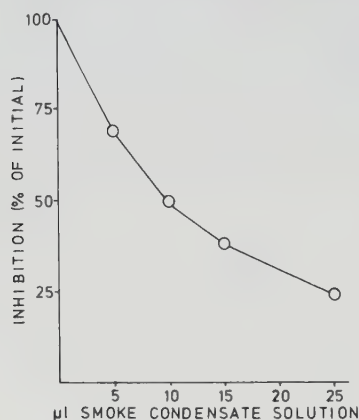


Figure 7. The inhibitory capacity of antileukoprotease tested after addition of increasing amounts (0–25 µl) of DMSO smoke condensate (20% w/v) solution and incubation for 18 h at 37°C. Controls with DMSO alone. Following incubation, the reaction mixtures were individually subjected to gel filtration on Sephadex G-25. 3.0 µg of the eluted peak was incubated with 5 µg elastase, and the esterolytic activity measured. The inhibiting capacity of antileukoprotease was unchanged after incubation with DMSO alone.

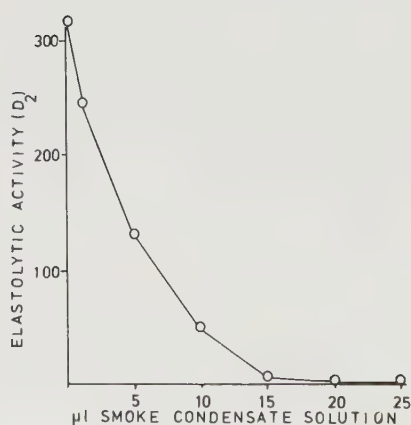


Figure 8. The elastolytic activity of granulocyte elastase (20 µg) after addition of increasing amounts of the DMSO solution of smoke condensate (0–25 µl). 10 µl samples (= 2 µg of elastase) of the reaction mixtures were immediately added to the elastin agarose plate which was incubated for 18 h at 37°C.

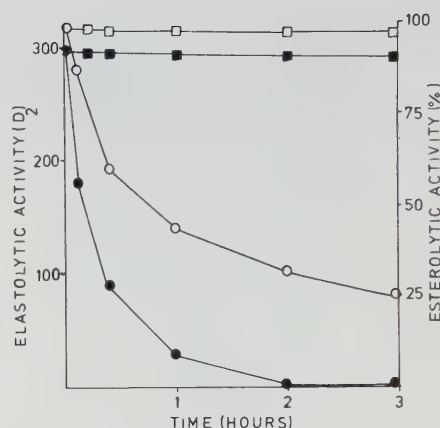


Figure 9. The elastolytic (●) and esterolytic (○) activity of granulocyte elastase (20 µg) after incubation with DMSO solution of smoke condensate (10 µl) for increasing time (0–3 h) at 37°C. Elastase mixed with DMSO without smoke condensate was incubated and analysed for reference; ■, elastolytic and ○, esterolytic activity. Before measuring the enzymatic activity, the reaction mixtures were subjected to gel filtration on Sephadex G-25.

yield a firmer basis for such conclusions.* It should be added that preliminary studies in our laboratory with aqueous solutions of cigarette smoke seem to demonstrate similar effects on the enzyme and inhibitors as the present extracts in DMSO.

Acknowledgments

We are indebted to Margareta Curvall at the Research Laboratory of the Swedish Tobacco Company for preparation of the cigarette smoke condensates used in this study.

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* Results made available to us while this paper was processed for publication indicate that cigarette smoking reduces the functional activity of α_1 -antitrypsin in bronchial lavage fluids in man [23] and rat [24] and possibly even in the blood of man [24]. The effect on proteases present in the lung was, however, not analysed in these studies.

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STUDIES ON THE ROLE OF ANTILEUKOPROTEASE
IN RESPIRATORY TRACT DISEASES

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Abstract. Antileukoprotease, an inhibitor of granulocyte elastase, was studied in paired sera from 19 patients with pneumonia and from 9 patients with cholecystolithiasis. The circulating level of antileukoprotease was significantly higher in patients with pneumonia compared with patients with cholecystolithiasis. In the latter group of patients, surgery raised the level of general acute-phase reactants, but did not affect the level of antileukoprotease. In sera from patients with pneumonia, antileukoprotease was recovered in a free form, as shown by gel-filtration. A local protective function of antileukoprotease is suggested by the finding that antileukoprotease was bound to granulocyte elastase in purulent bronchial secretions.

Key words. antileukoprotease - granulocyte elastase - pneumonia - acute-phase reactants - protease inhibitors

INTRODUCTION

Granulocyte elastase belongs to the proteolytic enzymes and is released in connection with cellular destruction and phagocytosis (18). Elastase attacks elastin (17) and can produce emphysema in experimental animals (11,22). It is present in purulent bronchial secretions (19,23), where it is the dominating cause of the elastolytic activity (19). A study of the concentration of elastase in serum has revealed a wide range of values. Patients with acute infections of the lower respiratory tract had, however, significantly higher circulating levels of elastase than normal subjects (24).

As a potentially dangerous enzyme, elastase is inactivated by strongly associated inhibitors (8). The two main inhibitors of elastase are the plasma protease inhibitor α_1 -antitrypsin and a low-molecular-weight inhibitor, named antileukoprotease (20). Antileukoprotease is located in the serous part of the sero-mucous glands in the respiratory tract mucosa (5,3) and in the uterine cervical mucosa (4). Antileukoprotease is responsible for about 90 % of the inhibitory capacity of bronchial secretions against elastase (20). Radioimmunoassay has shown a concentration of 125 $\mu\text{g/l}$ in sera of healthy blood donors (6).

The plasma protease inhibitors α_1 -antitrypsin and antichymotrypsin act, like orosomucoid, as general acute-phase reactants. Infectious diseases, as well as surgical trauma, result in an increased circulating level of these proteins (1). However, very little is known about the role of antileukoprotease in patients with acute respiratory diseases.

The purpose of the present study is to investigate the circulating level of antileukoprotease in serum in patients with acute respiratory tract diseases and to compare antileukoprotease with some general acute-phase reactants. The complex formation of antileukoprotease and elastase in serum and purulent bronchial secretions was studied to further ascertain the protective function and clinical significance of antileukoprotease.

MATERIALS

Clinical material

Serum samples were collected every two days from 19 adult patients with pneumonia (P-patients). "Convalescence sera" were obtained from 17 of these patients, at the earliest one month after dismissal from hospital. All patients had, on admission, a history of temperature $\geq 38^{\circ}$ C and coughing and their chest radiographs showed fresh pulmonary shadowing, consistent with acute lower respiratory infection.

Nine adult subjects with cholecystolithiasis, admitted for surgery (C-patients), were used as a control group. None of the control subjects had symptoms referable to the biliary tract or to acute disease at the time of operation. Serum samples were collected the day before and during three consecutive days after cholecystectomy. All the control subjects followed an uncomplicated post-operative course.

The serum samples were stored at -20° C until analysis, which was performed within four months.

Purulent bronchial secretions were collected from two groups of adult patients with chronic bronchitis, as defined by the presence of significant sputum production for at least three months of the preceeding two years (14). Four in-patients suffered an exacerbation of their condition, whilst in hospital. This was manifested by an increase in the symptoms of dyspnea, cough and sputum production, without parenchymal lung infiltrate on their chest radiographs.

Four male out-patients composed the other group. These subjects had chronic bronchitis, without exacerbation, during the period of study.

All sputum samples were collected in the morning and centrifuged within four hours at $240.000 \times g$ for three hours, according to Ryley & Brogan (21). The supernatants were stored at -20° C until examination, which was performed within 6 months.

Special materials

Sephadex G-75 was purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Agaros Seakem (batch 500 13) was obtained from Marine Colloids Div. FMC Corporation, Rockland, USA, and Suc(Ala)₃NA was a product from The Protein Research Foundation, Peptide Institute Inc., Osaka, Japan.

Antisera

Specific antisera against α_1 -antitrypsin, antichymotrypsin and orosomucoid are available at our laboratory.

METHODS

Sample analysis. All sera from the P-patients and C-patients were analysed for antileukoprotease, α_1 -antitrypsin, antichymotrypsin and orosomucoid. Sera from three of the P-patients were subjected to gel-filtration. The fractions were analysed for antileukoprotease and elastase.

The sol phase from sputa was tested for free elastase activity and subjected to gel-filtration and the eluted fractions analysed for the same proteins as described above.

Electroimmunoassays (12) were used for quantification of α_1 -antitrypsin, antichymotrypsin and orosomucoid.

Radioimmunoassays were used for the measurement of antileukoprotease and elastase (6,16).

Gel-filtration of serum and the sol phase of sputum was performed on a Sephadex G-75 column (0.9 x 60 cm), equilibrated with 0.05 M Tris-HCl buffer, pH 7.6, containing 0.6 M NaCl and 0.002 M CaCl₂.

Enzyme activity. Elastase activity and the inhibition of elastase by antileukoprotease was studied using Suc(Ala)₃NA as a substrate according to Bieth et al. (2).

Activity of antileukoprotease in serum was studied by adding elastase to the serum before subjecting the mixture to gel-filtration (6).

Statistical method. The difference between the groups were assessed by the Wilcoxon Rank-Sum Test for non-parametric data. The results of the differences within the groups were tested using the Wilcoxon Signed-Rank Test for paired differences. A two-tailed test was used for antileukoprotease and a one-tailed test for α_1 -antitrypsin, antichymotrypsin and orosomucoid.

RESULTS

Circulating levels of antileukoprotease, α_1 -antitrypsin, antichymotrypsin and orosomucoid in sera from P-patients and C-patients on different days are presented in Table I.

Antileukoprotease. The circulating level of antileukoprotease increased significantly, on admission to hospital, in the P-patients compared with pre-operative values of the C-patients ($p < 0.002$). The values decreased between day one and day five in the P-patients ($p < 0.02$). Convalescence sera displayed an additional decrease ($p < 0.002$) (Fig. 1). There was no significant difference between the convalescence values of the P-patients and the pre-operative values of the C-patients, which did not differ from healthy blood donors. The circulating level of antileukoprotease did not increase in the C-patients after cholecystectomy (Fig. 2). The distribution of antileukoprotease in serum of P-patients is shown in Fig. 3.

α_1 -Antitrypsin, antichymotrypsin and orosomucoid. In P-patients the circulating level was significantly increased for α_1 -antitrypsin ($p < 0.01$), antichymotrypsin ($p < 0.001$) and orosomucoid ($p < 0.001$) compared with the pre-operative normal values of the C-patients. The serum values of the P-patients increased during the first five days of their hospital stay and decreased to normal values in the convalescence sera (Fig. 1). The C-patients showed a significant increase in the circulating levels of α_1 -antitrypsin, antichymotrypsin and orosomucoid after cholecystectomy ($p < 0.01$) (Fig. 2).

Table 1. Serum concentrations in P-patients and C-patients of antileukoprotease, α_1 -antitrypsin, antichymotrypsin and orosomucoid. Normal ranges for the different proteins are as follow: antileukoprotease 100 - 150 $\mu\text{g/l}$; α_1 -antitrypsin 0.9 - 1.7 g/l ; antichymotrypsin 60 - 140 % of serum pooled from blood donors; orosomucoid 0.55 - 1.05 g/l . a) mean $\pm$ SD.

		Antileukoprotease		α_1 -Antitrypsin		Antichymotrypsin		Orosomucoid	
		mean circulating	range	mean circulating	range	mean % of	range	mean circulating	range
n		level $\mu\text{g/l}$ a)		level g/l a)		pooled sera	a)	level g/l a)	
P-patients									
day 1	19	453 $\pm$ 294	121-1320	2.99 $\pm$ 1.47	0.90-5.70	355 $\pm$ 154	160-702	1.72 $\pm$ 0.52	1.02-2.72
	3	372 $\pm$ 221	144-1000	3.01 $\pm$ 1.38	1.20-6.0	350 $\pm$ 148	142-680	1.58 $\pm$ 0.43	1.28-2.98
	5	332 $\pm$ 177	167-840	2.61 $\pm$ 0.94	1.35-4.80	344 $\pm$ 137	128-680	1.77 $\pm$ 0.54	1.02-3.06
Convalescence	17	147 $\pm$ 27	89-202	1.33 $\pm$ 0.56	0.60-2.70	107 $\pm$ 50	40-231	0.87 $\pm$ 0.30	0.58-1.45
C-patients									
preop.	9	127 $\pm$ 37	82-194	1.40 $\pm$ 0.53	0.90-2.0	98 $\pm$ 22	50-120	0.90 $\pm$ 0.26	0.61-1.31
postop.day 1	9	108 $\pm$ 30	65-145	1.50 $\pm$ 0.45	1.0 -2.40	167 $\pm$ 61	100-270	1.22 $\pm$ 0.60	0.61-2.26
	2	123 $\pm$ 46	82-203	1.99 $\pm$ 0.44	1.50-2.60	243 $\pm$ 83	140-370	1.56 $\pm$ 0.73	0.74-2.96
	3	128 $\pm$ 39	79-185	2.13 $\pm$ 0.45	1.40-2.70	247 $\pm$ 65	180-350	1.68 $\pm$ 0.51	1.22-2.70

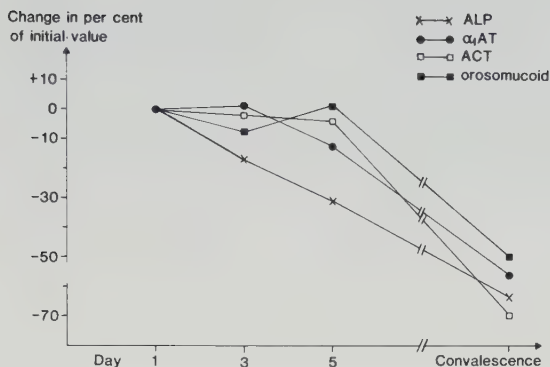


Fig. 1. Changes in serum concentrations of antileukoprotease (ALP), α_1 -antitrypsin (α_1 -AT), antichymotrypsin (ACT) and orosomucoid in patients with pneumonia. The values are calculated from the mean values in Table I.

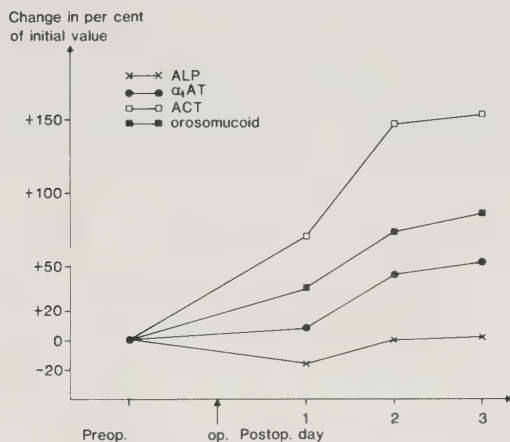


Fig. 2. Changes in serum concentration of antileukoprotease (ALP), α_1 -antitrypsin (α_1 -AT), antichymotrypsin (ACT) and orosomucoid in patients with cholecystolithiasis, pre- and post-operatively. The values are calculated from the mean values in Table I.

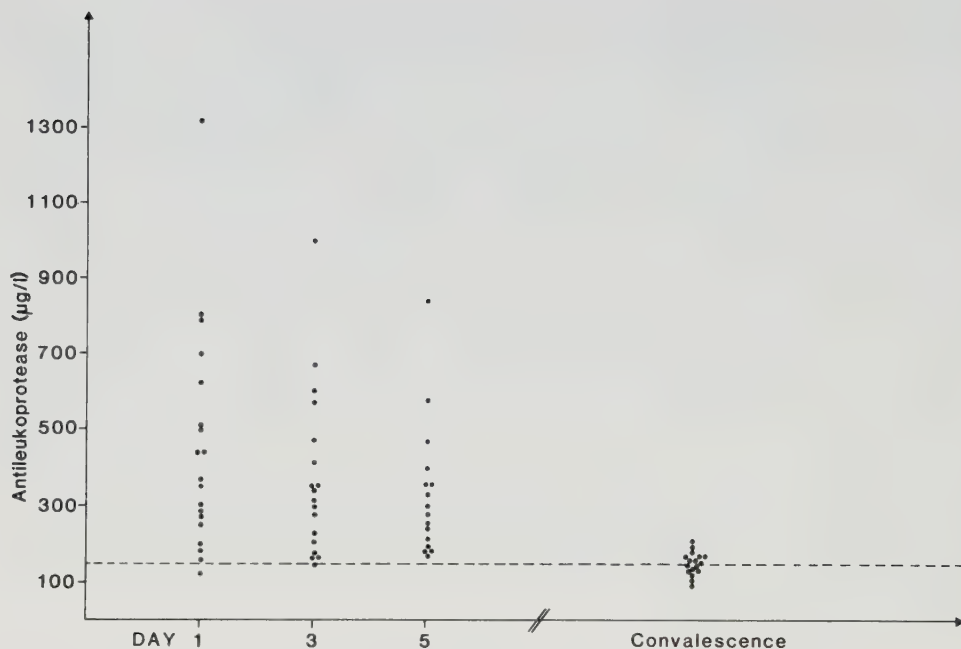


Fig. 3. Distribution of antileukoprotease in sera from patients with pneumonia. The interrupted line shows the upper range of the normal circulating value.

Gel-filtration of sera from patients with pneumonia. Antileukoprotease was eluted in a single peak, corresponding to the free inhibitor, by gel-filtration of sera on Sephadex G-75 (Fig. 4). It was active as an inhibitor as shown by complex formation with elastase after addition of elastase to serum. The immunoreactive elastase was eluted in the fractions corresponding to elastase- α_1 -antitrypsin complexes (Fig. 4).

Gel-filtration of sputa from patients with chronic bronchitis. The gel-filtrated sol phase of sputum from patients with chronic bronchitis eluted in different patterns. The two extremes are shown in Fig. 5 and Fig. 6.

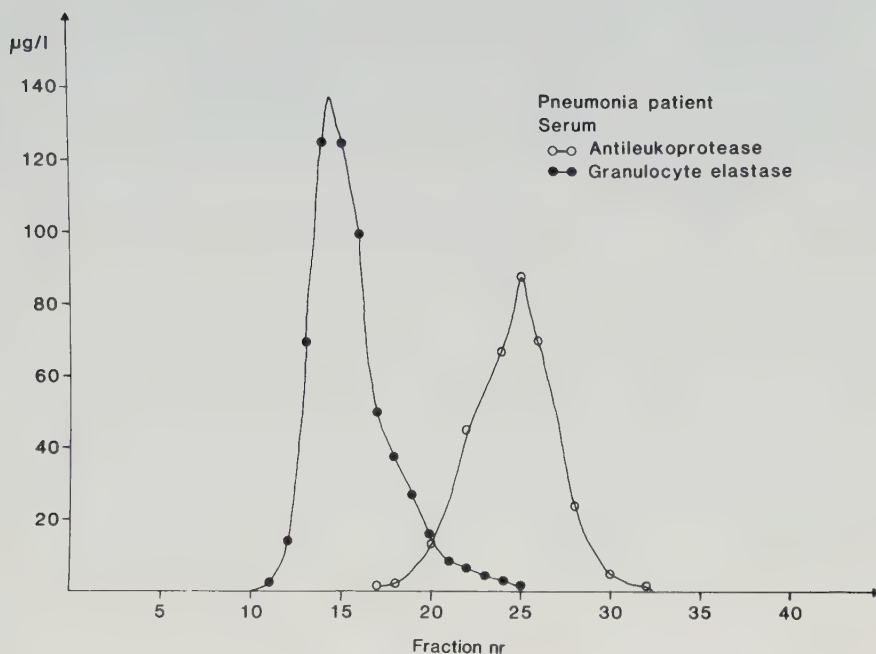


Fig. 4. Partition of antileukoprotease and granulocyte elastase in the fractions obtained by gel-filtration on Sephadex G-75 of serum from a patient with pneumonia. (For details; see methods).

Figure 5 represents the sol phase of sputum from a patient with exacerbation of chronic bronchitis. This sol phase exhibited free elastase activity. The peak of antileukoprotease corresponded to antileukoprotease-elastase complexes and the peak of elastase to free elastase.

The sol phase of sputum from an out-patient with chronic bronchitis is illustrated in Fig. 6. No free elastase activity was detectable. The dominating part of antileukoprotease was found in a free form and a small part was found in the fractions corresponding to antileukoprotease-elastase complexes. Elastase eluted in the fractions corresponding to elastase- α_1 -antitrypsin and elastase-antileukoprotease complexes.

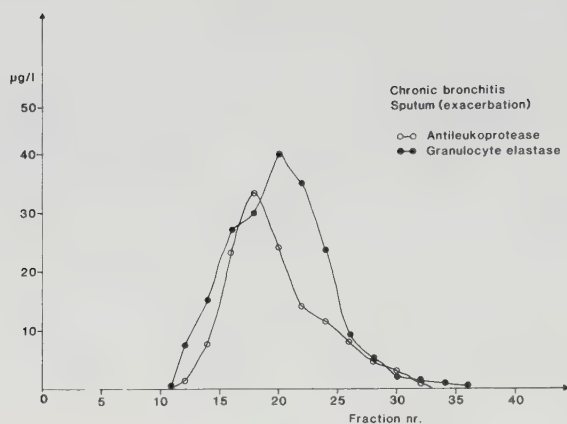


Fig. 5. Partition of antileukoprotease and granulocyte elastase in the fractions obtained by gel-filtration on Sephadex G-75 of the sol phase of sputum from a patient with chronic bronchitis (exacerbation). (For details; see methods).

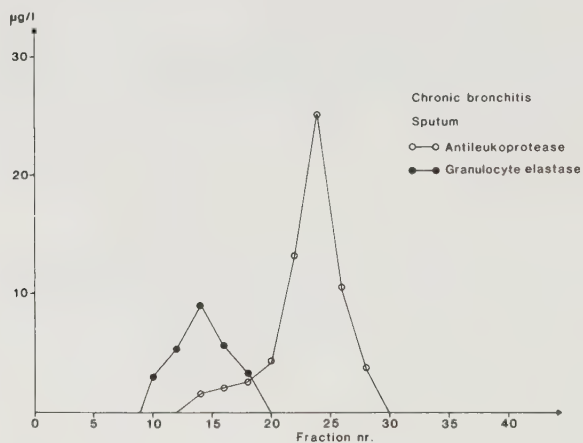


Fig. 6. Partition of antileukoprotease and granulocyte elastase in fractions obtained by gel-filtration on Sephadex G-75 of the sol phase of sputum from an out-patient with chronic bronchitis. (For details; see methods).

The sol phase of three out of eight sputa analysed, revealed free elastase activity. The gel-filtration analyses of these three samples showed no free antileukoprotease. The remaining five sputum samples were without elastase activity and the gel-filtration revealed the presence of free antileukoprotease. The samples recovered from the out-patients were included in the latter group.

DISCUSSION

An acute inflammatory disease in the respiratory tract, i.e. pneumonia, causes an increase in the production of the general acute-phase reactants, i.e. α_1 -antitrypsin, antichymotrypsin and orosomucoid. This is reflected in the serum (13). Antileukoprotease is normally present in serum as a trace protein (6), conceivably a spill-over from the respiratory tract. We have found a vast increase of antileukoprotease in the circulating level of patients with pneumonia but, as distinguished from these general acute-phase reactants, the serum level of antileukoprotease is significantly reduced on the fifth day. This suggests a close correlation between serum levels of antileukoprotease and the inflammatory process. The decrease may be explained by the different production sites of the proteins. Antileukoprotease has its production site in the respiratory tract mucosa (5), whereas α_1 -antitrypsin and orosomucoid are produced in the liver (13).

The circulating levels of the general acute-phase reactants are increased after surgical trauma (1). This is in contrast with antileukoprotease, which does not increase in serum after surgical trauma. The present results indicate that the increase of the serum level of antileukoprotease is a reaction to the infectious disease of the respiratory tract, and that antileukoprotease cannot be included among the general acute-phase reactants.

Previous investigations have shown antileukoprotease to form stable complexes with elastase (8). The complex can, however, be dissociated by α_1 -antitrypsin (8). In a recent study we found a freshly prepared equimolar complex of antileukoprotease and

elastase stable after gel-filtration, but dissociation was observed immediately after mixing the complex with serum (7). Elastase was recovered mainly in complex with α_1 -antitrypsin and to a minor degree in complex with α_2 -macroglobulin (7).

In line with these data, after gel-filtration of serum from patients with pneumonia, we have always obtained antileukoprotease in a free form and the immunoreactive elastase bound by α_1 -antitrypsin. The greatly increased circulating level of antileukoprotease in patients with pneumonia does not change this relationship.

The ability of antileukoprotease to act as a protection factor, was examined by studying the complex formation of enzyme and inhibitor close to the inflammatory process. Purulent bronchial secretions from patients with chronic bronchitis were used for this purpose. A wide range of proportions between different proteins in sputum, even in the same patient has been reported (21). The admixture of nasal secretions and saliva may be considerable in purulent bronchial secretions. In our study, we used purulent bronchial secretions without visible admixture of saliva, though the low content of antileukoprotease in mixed saliva would hardly disturb the results (15).

Purulent bronchial secretions usually exhibit free proteolytic activity (19). A major part of the elastase in these secretions is bound by insoluble substances in the gel phase (19). However, the sol phase of sputa from the in-patients with chronic bronchitis displayed high elastase activity. Antileukoprotease was recovered in complex with elastase and the excess of elastase was found in a free form. Although the measured level of antileukoprotease is high, it is nevertheless underestimated due to the changed immunoreactivity of antileukoprotease when it is in complex with elastase. About 30 % of the complexed antileukoprotease is accounted for with the radioimmunoassay for antileukoprotease (6).

Sputum with no elastase activity was found to contain an excess of free antileukoprotease and only a minor part bound by elastase. A previous study found elastase as well as α_1 -antitrypsin in low concentrations in sputum (23). The α_1 -antitrypsin content of these samples was not large enough to sufficiently

inhibit elastase (23). An excess of antileukoprotease may explain the lack of elastase activity. α_1 -Antitrypsin and antileukoprotease are not the only elastase inhibitors in bronchial secretions. The detection of another acid stable inhibitor of elastase in these secretions was recently reported (9). The urinary trypsin inhibitor is a relatively potent inhibitor of elastase and exhibits immunologic cross-reactivity with the inter- α -trypsin inhibitor (15) and preliminary data from our laboratory indicate that this inhibitor may be present in bronchial secretions.

Taken together the data presented support the assumption that antileukoprotease is a local inhibitor acting on the mucus membranes in the respiratory tract. The presence of antileukoprotease-elastase complexes in bronchial secretions, strongly indicates that antileukoprotease has a physiological function as a defence mechanism against the effects of granulocyte elastase. Analysis of antileukoprotease and elastase in the sol phase of sputum may yield important information as to the graveness of the respiratory tract disease. Furthermore, as antileukoprotease does not react as a general acute-phase reactant, the ability to determine and follow the circulating level of antileukoprotease in serum, may afford new opportunities in the clinical evaluation of patients with acute respiratory diseases.

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